VERIFICATION OF TRANSLATION

I, Mariko Ogura

of 6th Fl., Kantetsu Tsukuba-Science-City Bldg. 1-1-1, Oroshi-machi, Tsuchiura, Ibaraki, JAPAN

declare as follows:

1. That I am well acquainted with both the English and Japanese languages, and

2. That the attached document is a true and correct translation of a certified copy of the following application, which was made by me to the best of my knowledge and belief.

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Mariko Ogura
(Signature of Translator)
Mariko Ogura

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【Addressee】 Esq. Commissioner of the Patent Office

【Inventor】

【Address】 101 Makita, 15 Wakamiya-cho, Shinjuku-ku, Tokyo

【Name】 Ikuo Nishimoto

【Applicant】

【Identification Number】 598121341

【Name or Appellation】 KEIO UNIVERSITY

【Attorney】

【Identification Number】 100102978

【Patent Attorney】

【Name or Appellation】 Hatsushi Shimizu

【Nominated Attorney】

【Identification Number】 100108774

【Patent Attorney】

【Name or Appellation】 Kazunori Hashimoto

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[Document Name] Specification

[Title of the Invention] HUMANIN, A POLYPEPTIDE SUPPRESSING NEURONAL DEATH

[Claims]

[Claim 1] A polypeptide according to (a) or (b) shown below:

(a) a polypeptide having an amino acid sequence of SEQ ID NO: 4 or 6;

(b) a polypeptide having an amino acid of SEQ ID NO: 4 or 6, wherein one or several amino acids have been substituted, deleted, inserted, and/or added and the polypeptide retains functions equivalent to a polypeptide comprising an amino acid of SEQ ID NO: 4 or 6.

[Claim 2] A fusion polypeptide comprising the polypeptide of claim 1 fused with other polypeptides.

[Claim 3] A DNA encoding the polypeptide of claims 1 or 2.

[Claim 4] A vector into which the DNA of claim 3 is inserted.

[Claim 5] A host cell retaining the vector of claim 4.

[Claim 6] A method for producing the polypeptide of claim 1 or 2, comprising the steps of culturing the host cell of claim 5, and recovering the expressed polypeptide from the host cell or culture supernatant thereof.

[Claim 7] A method for suppressing neuronal death comprising the step of contacting a neuron with the polypeptide of claim 1.

[Claim 8] 13. A pharmaceutical composition comprising as the effective component the polypeptide of claim 1 or the vector of claim 4.

[Claim 9] The pharmaceutical composition of claim 8, wherein said composition is a neuronal death suppressant.

[Claim 10] The pharmaceutical composition of claim 8, which is used to prevent or treat diseases that are accompanied by neurodegeneration.

[Claim 11] The pharmaceutical composition of claim 8, which is used to prevent or treat Alzheimer's disease.

[Claim 12] An antibody that binds to the polypeptide of claim 1.

[Claim 13] A DNA for detecting or manipulating DNA encoding the polypeptide of claim 1, wherein the DNA comprises at least 15

nucleotides that are complementary to a DNA consisting of the nucleotide sequence of SEQ ID NO: 3 or to a complementary strand thereof.

[Claim 14] A method of screening for a chemical compound that binds to the polypeptide of claim 1, comprising the steps of:

(a) contacting a test sample with the polypeptide of any one of claims 1 to 4;

(b) detecting the binding activity between the test sample and the polypeptide; and

(c) selecting the compound that has the activity to bind to the polypeptide.

[Detailed Description of the Invention]

[0001]

[Technical Field of Industrial Application]

The present invention relates to polypeptides protecting neurons from cell death associated with Alzheimer's disease.

[0002]

[Prior Art]

Alzheimer's disease (AD) is currently the most actively studied neurodegenerative disease. AD is clinically characterized by progressive amnesia and cognitive impairment, and pathologically characterized by a wide range of neuronal loss, intraneuronal tangles, and extracellular senile plaques that have congophilic dense core. Effective treatment for AD still does not exist. It is generally accepted that not all, but many of the clinical manifestations of this disease are explained by progressive neuronal death. Elucidation of pathological mechanism of the onset of neuronal death in AD, to prevent AD is essential for developing novel treatments for AD.

[0003]

Four different groups of mutant genes are known to cause early-onset familial AD (FAD): V642I/F/G APP (the number represnts the position in APP_{695}, an APP consisting of 695 amino acids); KM595/596NL APP (NL-APP); presenilin (PS)-1 mutants; and PS-2 mutants. The present inventors have found evidence for the first time

suggesting that these FAD genes might cause cell death of neurons, based on the observation on nerve cell line F11 wherein three V642 type mutant cDNA of APP was transiently expressed (Yamatsuji, T. et al. (1996) Science 272, 1349-1352). The result was also confirmed by experiments conducted by other group which used primary cultured neurons and immortalized nerve cell lines (Zhao, B. et al. (1997) J. Neurosci. Res. 47, 253-263; Bursztajn, S. et al. (1998) J. Neurosci. 18, 9790-9; Luo, J. J. et al. (1999) J. Neurosci. Res. 55, 629-42). Further, Wolozin et al. revealed that FAD-linked mutant N141I PS-2 significantly enhances cell mortality in PC12 cells(Wolozin, B. et al. (1996) Science 274, 1710-1713), and that FAD-linked mutant PS-1 induces apoptosis of T lymphocytes (Wolozin, B. et al. (1998) Neurobiol. Aging 19, S23-27). Although it is controversial whether the mutant PS-1 is a direct stimulation factor of neuronal death (Weihl, C. C. et al. (1999) J. Neurosci. 19, 5360-9) or has no effect on neuronal death (Bursztajn, S. et al. (1998) J. Neurosci. 18, 9790-9), it is obvious that FAD-linked mutants of APP and PS-2 induce neuronal cell death. Furthermore, regarding PS-1, enhanced sensitivity to neuronal death induced by Aβ addition and/or trophic factor deficiency due to the expression of PS-1 mutant (Guo, Q. et al. (1996) Neuroreport 8, 379-83; Zhang, Z. et al. (1998) Nature 395, 698-702; Guo, Q. et al. (1999) Proc. Natl. Acad. Sci. U.S.A. 96.,4125-30); and enhanced sensitivity to neuronal death by trophic factor deficiency in cultured cortical neurons, derived from transgenic rats overexpressing wild-type PS-1, compared with those in non-transgenic controls (Czech, C. et al. (1998) Neuroscience 87, 325-36); and such have been repeatedly observed. Therefore, it is highly possible that all of the four types of known FAD genes (V642-type mutant APP, NL-APP, PS-1 mutant, and PS-2 mutant) induce neuronal death or amplify the vulnerability of neurons to other cell death stimuli under certain conditions. Therefore, finding molecules that suppress AD gene-induced cell death observed in cultured neurons is suggested to be the most important key for developing methods to treat AD.

[0004]

[Problems to Be Solved by the Invention]

An object of the present invention is to provide polypeptides

that protect neurons from cell death associated with Alzheimer's disease, and use of the same.

[0005]

[Means to Solve the Problems]

The present inventor has previously established a nerve cell line (F11/EcR/V642I), which inductively expresses familial Alzheimer's disease-type mutant V642I amyloid precursor proteins (V642I APP) (see International Publication No. PCT/JP99/04771). According to the system, V642I APP is expressed in F11 neurons in response to ecdysone treatment. Cell death occurred in almost all of the F11/EcR/V642I cells incubated with ecdysone for 2 to 3 days; whereas cell death occurred in only a few cells in the control incubation. The present inventor used the F11/EcR/V642I cells to search for genes that act as antagonists of V642I APP-induced neuronal death.

[0006]

A cDNA library was constructed from the brain of Alzheimer's disease (AD) patient, and was transfected into the F11/EcR/V642I cells mentioned above. Then a death trap screening operation was repeatedly performed to select cells that survived neuronal death induced by V642I APP. As a result, the present inventor succeeded in identifying a novel gene that protect cells against neuronal death induced by V642I APP. It was revealed that the clone, dubbed Humanin cDNA, encoding a novel polypeptide of 24-amino acids, suppresses neuronal death associated with AD. That is, the clone rescued neuronal death induced by V642I APP, KM595/596NL APP, M146L presenilin (PS)-1, and N141I PS-2. In contrast, the clone had no effect on neurotoxicity of polyglutamine repeat Q79, associated with Huntington's disease/ spinocerebellar ataxia (HD/SCA); and mutants of SOD1 associated with familial amyotrophic lateral sclerosis (FALS). Transfection of HN cDNA into neurons led to transcription and production of expected peptides, which peptides were secreted into the culture supernatant up to a level of about 10 μM. The culture supernatant was enough active to demonstrate significant protection of cells from neuronal death induced by V642I APP. Synthetic Humanin polypeptide also showed neuroprotective action with similar

dose-response properties against the four types of AD genes, and its suppression was maximal at 1 to 10 μM. Cys at position 8 and Ser at position 14 were found to be important according to an experiment detecting the activity of polypeptides with modified structure. C8A substitution completely deprived the polypeptide of the cell death rescue activity. On the other hand, S14G substitution remarkably enhanced the rescue activity of the polypeptide. S14G polypeptides showed complete protective action against all of the four types of AD genes at 1 to 10 nM. Anti-AD activity of Humanin was also observed in primary cultured cortical neurons. Specifically, μM levels of Humanin and nM levels of S14G derivatives protected cells form cell death and cell damage caused by Aβ, whereas C8A lacked such activity. These novel polypeptides open a new path to develop therapeutic drugs for AD, and at the same time, are expected to contribute greatly to the development of AD therapy aiming at protection of neurons from cell death.

[0007]

The present invention relates to novel polypeptides that protect cells from neuronal death associated with AD, and use of the same. More specifically, the present invention relates to:

(1) a polypeptide according to (a) or (b) shown below:

(a) a polypeptide having an amino acid sequence of SEQ ID NO: 4 or 6;

(b) a polypeptide having an amino acid sequence of SEQ ID NO: 4 or 6, wherein one or several amino acids have been substituted, deleted, inserted, and/or added and the polypeptide retains functions equivalent to a polypeptide comprising an amino acid sequence of SEQ ID NO: 4 or 6;

(2) a fusion polypeptide comprising the polypeptide of (1) fused with other polypeptides;

(3) a DNA encoding the polypeptide of (1) or (2);

(4) a vector into which the DNA of (3) is inserted;

(5) a host cell retaining the vector of (4);

(6) a method for producing the polypeptide of (1) or (2), comprising the steps of culturing the host cell of (5), and recovering the expressed polypeptide from the host cell or culture supernatant

thereof;

(7) a method for suppressing neuronal death comprising the step of contacting a neuron with the polypeptide of (1);

(8) a pharmaceutical composition comprising as the effective component the polypeptide of (1) or the vector of (4);

(9) the pharmaceutical composition of (8), wherein said composition is a neuronal death suppressant;

(10) the pharmaceutical composition of (8), which is used to prevent or treat diseases that are accompanied by neurodegeneration;

(11) the pharmaceutical composition of (8), which is used to prevent or treat Alzheimer's disease;

(12) an antibody that binds to the polypeptide of (1);

(13) a DNA for detecting or manipulating DNA encoding the polypeptide of (1), wherein the DNA comprises at least 15 nucleotides that are complementary to a DNA consisting of the nucleotide sequence of SEQ ID NO: 3 or to a complementary strand thereof; and

(14) a method of screening for a chemical compound that binds to the polypeptide of (1), comprising the steps of:

(a) contacting a test sample with the polypeptide of any one of (1) to (4);

(b) detecting the binding activity between the test sample and the polypeptide; and

(c) selecting the compound that has the activity to bind to the polypeptide.

[0008]

[Mode for Carrying Out the Invention]

The present invention provides polypeptides which protect neurons from cell death associated with Alzheimer's disease. The amino acid sequence of Humanin polypeptide, isolated by the present inventor, is indicated in SEQ ID NO: 4, and the cDNA sequence of the open reading frame encoding the polypeptide is indicated in SEQ ID NO: 3. Humanin antagonizes neuronal death associated with AD, and shows a saturation activity at a concentration of about 10 μM. In addition, HNG (S14G) (SEQ ID NO: 6), which is a Humanin with an amino acid substitution, showed 100 to 1000 fold higher antagonizing effect compared to Humanin. The polypeptide of the present invention

includes Humanin and HNG.

[0009]

Further, the present invention demonstrated that addition of a FLAG tag (DYKDDDDK) to the C-terminus of Humanin does not affect the neuroprotective action thereof (Example 3). Furthermore, even when the four C-terminal amino acids (KRRA) of Humanin were substituted with other amino acids, a neuroprotective action equivalent to that of the original Humanin was present in the substituted polypeptide (Example 5). These facts demonstrate that polypeptides with equivalent or higher neuroprotective action to Humanin or HNG can be prepared by introducing mutations to the amino acid sequence of Humanin or HNG. Therefore, the polypeptide of the present invention includes a polypeptide having an amino acid sequence of Humanin (SEQ ID NO: 4) or HNG (SEQ ID NO: 6), in which one or several amino acid is substituted, deleted, inserted, and/or added and the polypeptide has functions equivalent to Humanin or HNG. The phrase "have functions equivalent to Humanin of HNG" means that a polypeptide of interest has an activity for suppressing neuronal death associated with AD. Neuronal death associated with AD is induced by the expression of APP, PS-1, or PS-2 mutants (for example, V642I APP, NL-APP, M146L PS-1, and N141I PS-2) in established neuronal cell lines (for example, F11 cells) and primary neuronal cultures (for example, rat brain cortical primary culture); and also by the addition of Aβ (for example, Aβ1-43) to primary neuronal cultures. The polypeptides of the present invention include those that suppress at least any one of these neuronal deaths associated with AD. The suppression of cell death doesn't have to be a complete suppression so long as the suppression is significant. The activity of proteins to suppress neuronal death can be detected according to the method described in the Examples, or by other published methods (see for example, International Publication No. PCT/JP99/04771).

[0010]

The polypeptide having a mutation in amino acids may be produced as a synthetic polypeptide by known peptide synthesis techniques (Japanese Biochemical Society edition, "Shin Seikagaku Jikken Koza Tanpakushitu (New Course on Biochemistry Experiments, Proteins) VI,"

pp. 3-74, Tokyo Kagakudojin, 1992). The method for peptide synthesis may be either solid-phase synthesis or liquid-phase synthesis. Further, polypeptides with arbitrary amino acid mutations can be prepared through the introduction of mutation to Humanin cDNA (for example, SEQ ID NO: 3) by the production of synthetic DNA or by site directed mutagenesis; and then, expressing the mutated cDNA in a host cell. There are no limitations on the number and position of the amino acids to be modified so long as the obtained polypeptide has functions equivalent to Humanin of HNG.

[0011]

Furthermore, the polypeptides of this invention include salts thereof. Such salts are derived from acids or bases of the polypeptides. Specifically, such salts can be exemplified by salts formed with inorganic acids (for example, hydrochloride, phosphate, hydrobromide, hydrosulfate, nitrate, etc.); salts formed with organic acids (for example, acetate, lactate, formate, butyrate, glycolate, propionate, fumarate, maleate, succinate, tartrate, citrate, malate, oxalate, benzoate, methane sulfonate, benzene sulfonate, etc.); and salts formed with bases (for example, ammonium salt, alkali metal salts such as sodium salt and potassium salt, alkaline earth metal salts such as calcium salt and magnesium salt, and salts formed with organic bases, and salts formed with amino acids such as arginine and lysine).

[0012]

Furthermore, the polypeptide of the present invention includes derivatives thereof. Herein, the term "derivatives" refers to molecules that have a form, which has been altered by modification, addition, mutation, substitution, or deletion of functional groups of the polypeptide of this invention according to conventional methods. Such alterations of functional groups are carried out, for example, to protect functional groups of the polypeptides, to regulate the stability or histological localization of the polypeptides, or to regulate the activity of the polypeptides, and so on. The polypeptides of the present invention are exemplified by those polypeptides wherein any one of the N-terminus, C-terminus, and functional groups of the polypeptides constituting amino acid side

chains are modified by substituents, such as protecting groups. The substituents include, for example, various alkyl groups, acyl groups, amide groups, phosphate groups, amino groups, carboxyl groups, and ester groups; however, the present invention is not limited to these examples.

Furthermore, the polypeptides of the present invention include polymers, such as dimers wherein the polypeptides are bound to each other; branched molecules; and cyclized molecules. Further, the polypeptides may be bound to a carrier.

[0013]

Amino acids that constitute the polypeptides of the present invention may be in the L form and/or D form. The use of D amino acids is effective for lowering degradation by peptidases. Additionally, the amino acids are not limited to natural amino acids, and may be also unnatural amino acids. Unnatural amino acids are exemplified by homoserine, β-hydroxyvaline, 0-4-hydroxyphenyl tyrosine, α-t-butyl glycine, 2-amino butyrate, α-cyclohexyl glycine, α-phenyl glycine, and such. Further, the peptide bonds of the polypeptides may be appropriately substituted with covalent bonds other than peptide bonds . The sensitivity to peptidases of the polypeptides can be lowered by the substitution to non-peptide bonds, which enhances drug efficacy duration and which offers a wide selection of administration routes. The non-peptide bonds are exemplified by imino bonds, ester bonds, hydrazine bonds, semicarbazide bonds, and azo bonds, but the present invention is not limited to these examples.

Further, chemical compounds, that mimic the structure of the polypeptides of the present invention, may be designed. For example, based on the physical and chemical properties (which may be analyzed by conventional methods including active site modification, NMR, and X-ray crystallography) relating to the structure of the polypeptides of this invention a map of physical and chemical functions, that are important for neuroprotective action of the polypeptides, is constructed. Then, molecules that simulate these functions are designed and synthesized. Alternatively, the polypeptides of the present invention are expected to bind to a receptor due to its high activity, and thus, compounds that bind to the same receptor may be

designed. Whether molecules derived in this manner possess a neuroprotective action or not can be assayed according to the method described in the Examples.

[0014]

The present invention also provides DNA encoding a polypeptide of this invention. There is no particular limitation on the origin of the DNA of the present invention, and includes synthetic DNA, genomic DNA, cDNA, and such. The DNA of this invention includes a cDNA that encodes Humanin, described in SEQ ID NO: 3. A DNA having any nucleotide sequence based on the degeneracy of genetic code may be included so long as it encodes the amino acids described in SEQ ID NO: 4 or 6.

The DNA of the present invention may be used to produce a polypeptide of this invention by inserting the DNA into a vector. Furthermore, it is also possible to use the DNA for application to gene therapy as described below.

The host-vector system used for producing the polypeptides of the present invention may be the baculovirus-Sf cell line (Okamoto et al., J. Biol. Chem. 270: 4205-4208, 1995); the pcDNA-CHO cell line (Takahashi et al., J. Biol. Chem. 270: 19041-19045, 1995); the CMV promoter plasmid-COS cell line (Yamatsuji et al., EMBO J. 15: 498-509, 1996); and such, but are not limited thereto.

[0015]

The polypeptides of the present invention may be secreted from host cells. As described in the Examples, Humanin, HNG, and such were secreted from cells, wherin the polypeptides were expressed to the extracellular region, and the secreted polypeptides antagonized neuronal death. When secreted to the cell exterior, the polypeptides of the present invention can be conveniently recovered from the culture supernatant of the host cells.

[0016]

The present invention also provides pharmaceutical compositions containing a polypeptide of this invention, or a vector, wherein a DNA encoding a polypeptide of this invention has been inserted, as its active ingredient. A polypeptide of the present invention can protect cells from neurodegeneration by adding the

polypeptide extracellularly, or by intracellular expression of the polypeptide. Therefore, a polypeptide of this invention is useful as a pharmaceutical composition particularly active against diseases associated with neurodegeneration.

[0017]

As described in the Examples, chemically synthesized Humanin (HN) polypeptide suppresses neuronal death at a concentration of about 10 nM or more in the extracellular solution, and a maximum suppression is achieved at a concentration of 1 to 10 μM. On the other hand, HNG polypeptides showed maximum suppression activity at about 10 nM or less. The neuroprotective action is presented by introducing and expressing a DNA encoding the polypeptides in the cell. Therefore, a vector expressing a polypeptide of this invention as a medicament may be used to perform gene therapy. Administration methods for the vectors include *in vivo* and *ex vivo* methods. Vector systems for gene therapy include: adenovirus vector; AAV (adenovirus-associated virus) vector; herpesvirus vector (all refer to Robbins and Ghivizzani, Pharmacol. Ther. 80: 35-47, 1998); retrovirus vector (Engel and Kohn, Front. Biosci. 4: e26-33, 1999); lentivirus vector (Lundstrom, K., 1999, J. Recept. Signal. Transduct. Res. 19: 673-686); and such, but are not limited thereto.

[0018]

The target diseases to be prevented or treated using a polypeptide of the present invention, or using a vector that expresses the polypeptide is not limited in any way, so long as the used polypeptide of the present invention is effective for treating the disease. Examples of preferred target diseases include neuron-related diseases, in particular Alzheimer's disease. Previous studies have revealed that cell death of neurons occurs in Alzheimer's disease (I. Nishimoto et al., 1997, Adv. Pharmacol., 41: 337-368). Some sort of activation of APP (I. Nishimoto et al., 1998, Neurobiol. Aging., 19: S33-S38) and presenilin (Nishimura et al., 1999, Clin. Genet. 55: 219-225) are suggested to be associated with the cell death. Therefore, pharmaceutical compositions of this invention are expected to be applicable as medicament for protection against neurodegeneration that occurs in Alzheimer's disease. In

addition to Alzheimer's disease, for example, diseases caused by cell death of neurons due to cerebral ischemia (T. Kirino, 1982, Brain Res., 239: 57-69) can be prevented by the use of a pharmaceutical composition of the present invention. Further, Parkinson's disease that accompanies dementia (M.H. Polymeropoulos et al., 1997, Science, 276: 2045-2047); diffuse Lewy bodies disease (M.G. Spillantini et al., 1998, Proc.Natl.Acad.Sci. USA, 95: 6469-6473); dementia that accompanies Down's disease; and such are also targets of the treatment and prevention using a protein of the invention. Furthermore, since APLP1, which is an APP analogue, is said to be the causative gene for congenital nephrotic syndrome (Lenkkeri, U. et al., 1998, Hum. Genet. 102: 192-196), renal diseases, such as nephrotic syndrome, is also the target for the treatment and prevention.

[0019]

In addition to the direct administration of the active ingredient to a patient, a pharmaceutical composition of this invention may be formulated following conventional drug implementations. For example, the composition may be administered after appropriately formulating it with pharmacologically acceptable carriers or medium, specifically, sterilized water or saline, vegetable oils, emulsifiers, suspending agents, detergents, stabilizers, sustained-release preparations, and such.

[0020]

Administration to patients may be carried out depending on the properties of the used active ingredient. Example of suitable administration methods include percutaneous, intranasal, transbronchial, intramuscular, intraperitoneal, intravenous, intraspinal, intracerebroventricular, or oral administrations, but are not limited thereto. When using the pharmaceutical composition in the treatment of cerebral neurodegenerative diseases, it is preferable to introduce the pharmaceutical composition to the central nervous system by an appropriate arbitrary route including a intravenous, intraspinal, intracerebroventricular, or intradural injection. The dosage varies according to the age, body weight, condition of a patient, method of administration, and such, but one skilled in the art can suitably select them. The dosage and

administration method varies depending on the histological localization of the active ingredient of the pharmaceutical composition of the present invention, therapeutic purpose, body weight, age, and condition of a patient, and such, but can be selected suitably by those skilled in the art.

[0021]

For example, to protect cerebral neurons against degeneration in Alzheimer's disease treatment, it is preferable to administer a polypeptide of the present invention so that the concentration around the target cells is sufficient to effectively suppress neurodegeneration. Specifically, Humanin polypeptide or compounds, that have equivalent protective action against neuronal death with Humanin, should be administered at a concentration of at least 1 nM or more, preferably 10 nM or more, more preferably 100 nM or more, and much more preferably 1 μM or more. HNG or comounds, that have equivalent protective action against neuronal death with HNG, should be administered at a concentration of at least 1 pM or more, preferably 10 pM or more, more preferably 100 pM or more, and much more preferably 1 nM or more. The dosage to achieve these concentrations can be appropriately determined taking the administration route into consideration.

[0022]

The present invention also provides antibodies binding to a polypeptide of the invention. The antibodies of this invention include polyclonal antibodies and monoclonal antibodies. Polyclonal antibodies can be prepared, for example, as follows: Humanin and HNG, or partial peptides thereof are prepared; rabbit, goat, sheep, and such are sensitized with these peptides as the antigen. Antigenic peptides can be bound to other proteins according to needs. For example, they can be bound with carrier proteins, such as key-hole limpet hemocyanin and albumin for immunization. Monoclonal antibodies can be prepared using splenocytes of immunized mice and rats to obtain hybridomas that produce monoclonal antibodies. Production of antibodies can be carried out according to conventional methods (Ed. Harlow and David Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

Using conventional biochemical techniques, such as ammonium sulfate fractionation, protein G sepharose column, and affinity column with immobilized antigens, polyclonal antibodies can be purified from serum, and monoclonal antibodies can be purified from hybridoma culture supernatant or from ascites of animals inoculated with hybridoma.

Additionally to the use of the antibodies prepared in this manner to adsorb the polypeptide of this invention, the antibodies may be utilized, for example, to test and diagnose structural alterations of a polypeptide of this invention, and to detect the expression level of a polypeptide of this invention.

[0023]

Decrease in blood or interstitial concentration of Humanin or Humanin-like peptide, including concentration in the nerve tissue, may be used to diagnose or prognosticate degenerative diseases of the nerves, including AD, and degenerative diseases of other organs. For example, it is possible that the progress of the disease of AD patients with low HN activity in blood is faster with bad prognosis compared to patients of the same kind of AD with high HN activity. Conceivable methods for testing are exemplified by measuring the concentrations of a pepetide of the invention in the blood or tissue samples by RIA using anti-Humanin antibody, or by testing biopsy samples by immunohistological staining. Furthermore, for example, the polypeptide level can be monitored during the treatment which includes administration of a polypeptide of this invention.

[0024]

The antibodies of the present invention may be antibody fragments thereof, so long as it binds to a protein of the invention. For example, the antibody fragment may be Fab, $F(ab')_2$, Fv, or modified antibodies thereof. Additionally, humanized antibodies or human antibodies, and such are also included in the antibodies of the present invention.

[0025]

The present invention also provides a DNA comprising at least 15 nucleotides, which is complementary to a DNA (SEQ ID NO: 3) encoding Humanin or complementary strand thereof, and are used to detect or

manipulate a gene encoding a polypeptide of this invention. The term "detection or manipulation of a gene" includes detection and regulation of the expression of a gene. Herein, the term "complementary strand" is defined as one strand of a double strand DNA composed of A:T and G:C base pair to the other strand. Also, "complementary" is defined as not only those completely matching within a continuous region of at least 15 nucleotides, but also those having a homology of at least 70%, preferably at least 50%, more preferably 80%, even more preferably 90% or more (for example, 95% ore more) within that region. The homology may be determined, for example, according to a method described in the literature (Altschul, S. F. et al., 1990, J. Mol. Biol. 215: 403-410).

[0026]

Such DNA includes, probes and primer for detecting or amplifying DNAs or RNAs encoding a peptide of the invention; as well as nucleotide and nucleotide derivatives (for example, antisense oligonucleotides, DNAs encoding ribozymse, and such) for suppressing the expression of a polypeptide of this invention. When used as a primer, such a DNA is complementary at the 3'-end, and restriction enzyme recognition sequences or tags can be added to the 5'-end.

[0027]

Further, the present invention provides a method of screening for compounds that bind to a polypeptide of the invention. Such screening can be performed by a method comprising the steps of: (a) contacting a polypeptide of the present invention with a test sample; (b) detecting the binding activity between the polypeptide of the invention and the test sample; and (c) selecting the sample that bind to the polypeptide of this invention.

[0028]

Depending on the screening method, a polypeptide of the invention may be used in the screening as a soluble polypeptide, or in a form bound to a support. A polypeptide of the invention may be labeled. Examples of labeling include labeling by radioactive isotopes, fluorescent substances, and biotin or digoxigenin; tag sequence addition; and such.

[0029]

Test samples for the screening may be, for example, purified proteins (including antibodies); expression products of gene libraries; synthetic peptide libraries; cell extracts; cell culture supernatants; libraries of low-molecular weight synthetic compounds; natural materials, such as soil; solutions containing substances released from bacteria, such as Actinomyces broth; and so on, but are not limited thereto. Test samples to be used in the screening may be appropriately labeled according to needs. The labels include, for example, radioactive labels, fluorescent labels, and such, but are not limited thereto.

[0030]

For example, screening for proteins, that bind to a polypeptide of the present invention, can be carried out by applying cell extract of tissues or cells, expected to express proteins that bind to a polypeptide of this invention, to an affinity column to which a polypeptide of this invention is immobilized; and purifying the proteins that specifically bind to the column.

[0031]

Alternatively, a cDNA library is prepared from tissues or cells, expected to express proteins that bind to a polypeptide of the invention (for example, brain cortical tissue; and neurons, such as F11) using phage vectors; then plaques are formed on agarose; and screening by Western blotting is carried out using labeled polypeptides of this invention. The screening can be also conducted by a "two hybrid system", and so on. Specifically, a method utilizing a "two hybrid system" is conducted as follows: (1) a DNA-binding peptide, such as GAL4 DNA-binding region, and a transcription activating peptide, such as GAL4 transcription activation region, is expressed as a fusion protein with a polypeptide of the present invention and a test protein, respectively; and (2) the binding of the protein of the present invention and the test protein is detected as the expression of a reporter gene attached downstream of a promoter, having a binding sequence of the DNA binding peptide.

[0032]

Furthermore, receptors of a polypeptide of the present invention can be cloned by the screening method of this invention.

In case of screening receptors, it is preferable to prepare the test samples from tissues or cells, expected to express receptors (for example, brain cortical tissue, nerve cell line, neuroblastoma cells, and teratocarcinoma cells). Examples of nerve cell lines include F11 cells; PC12 cells (L. A. Greene and A. S. Tischler, 1976, Proc. Natl. Acad. Sci. USA, 73: 2424-2428); NTERA2 cells (J. Skowronski and M. F. Singer, 1985, Proc. Natl. Acad. Sci. USA, 82: 6050-6054); SH-SY5Y cells (L. Odelstad et al., 1981, Brain Res., 224: 69-82); and so on.

[0033]

Alternatively, molecules binding to a polypeptide of the present invention can be screened by contacting synthetic compounds; natural product bank; and random phage peptide display libraries to an immobilized polypeptide of the invention. Further, screening by detecting the binding utilizing surface plasmon resonance imaging (for example, manufactured by BIAcore) is possible. These screening methods may be performed by high-throughput screening utilizing combinatorial chemistry techniques.

Compounds, that bind to a polypeptide of the present invention, obtained according to the screening method of this invention, serve as candidate compounds, regulating the activity of a polypeptide of the invention. Thus, these compounds are applicable to prevent or treat Alzheimer's disease.

[0034]

[Examples]

The present invention will be described in detail using Examples below, but the invention is not to be construed as being limited to these Examples. The experimental procedures described in these Examples are as follows:

V642I APP cDNA has been described previously (Yamatsuji, T. et al. (1996) Science 272, 1349-1352). The M146L mutant of PS-1 cDNA and the N141I mutant of PS-2 cDNA were gifts from Dr. Peter St. George-Hyslop (Sherrington, R. et al. (1995) Nature 375, 754-760) and Dr. Luciano D'Admio (Wolozin, B. et al. (1996) Science 274, 1710-1713), respectively. All of the FAD genes used in the Examples were encoded in pcDNA vectors (Funk, C.D. et al. (1990) Proc. Natl. Acad. Sci. USA, 87: 5638-5642). The FALS-associated mutant of SOD1

cDNA (A4T, G85R, G93A) (Takahashi, H. et al. (1994) Acta Neuropathol. 88, 185-8), and pDN-E/G5H-Q79 were gifts from Dr. Shoji Tsuji, (Niigata University School of Medicine, Niigata, Japan), and Dr. Akira Kakizuka (Osaka Biomedical Research Center, Osaka, Japan), respectively. The pHN plasmids encoding Humanin were constructed by inserting Humanin DNAs into the polycloning site of pFLAG-CMV-5a vectors (pFLAG) (Eastman Kodak). Briefly, pFLAG-CMV-5a plasmids were digested with *Eco*RI and *Kpn*I, and the Humanin-encoding sense primer (5′-AATTCACCATGGCTCCACGAGGGTTCAGCTGTCTCTTACTTTTAACCAGTGAAATTGACC TGCCCGTGAAGAGGCGGGCAGGTAC-3′/ SEQ ID NO: 1) and antisense primer (5′-CTGCCCGCCTCTTCACGGGCAGGTCAATTTCACTGGTTAAAAGTAAGAGACAGCTGAACC CTCGTGGAGCCATGGTG-3′/ SEQ ID NO: 2) were ligated. The plasmid expresses Humanin polypeptide fused with FLAG tag (DYKDDDDK) to the C-terminus. The pFLAG plasmids (pHNG and pHNA), encoding mutant HN, were constructed from pHN using Quick Change Site-directed Mutagenesis Kit (Stratagene). The sequence was confirmed by direct sequencing. Synthetic HN polypeptides (sHN) and structurally modified synthetic polypeptides synthesized in Peptide Inc. and purified to 95% or higher purity were used. Anti-FLAG antibody was purchased from Eastman Kodak (M2 monoclonal antibody, Cat. #IB13010). Aβ1-43 was purchased from BACHEM (Cat. #H-1586). Other reagents were all commercially available.

[0035]

F11 cells described previously (Platika, D. et al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 3499-3503) were cultured in HamF-12 media containing 18% fetal bovine serum (FBS) and antibiotics. $7x10^4$/well of F11 cells were seeded into a 6-well plate; cultured in HamF-12 containing 18% FBS for 12 to 16 hours; transfected 3 hours with plasmids encoding FAD genes and plasmids encoding HN by lipofection in the absence of serum (1 μg of FAD cDNA expression plasmid, 1 μg of HN cDNA expression plasmid, 4 μl of LipofectAMINE, 8 μl of Plus reagent); and then, were cultured for 2 hours in HamF-12 media containing 18% FBS. Then, culture media were exchanged with HamF-12 media containing 10% FBS; and the cells were cultured for additional 67 hours. 72 hours after transfection, cell death was measured by

trypan blue exclusion assay. Experiments using synthetic HN polypeptides were conducted as follows: F11 cells (at $7x10^4$/well in a 6-well plate) were transfected 3 hours with FAD genes in the absence of serum, as mentioned above; after cultivation for 2 hours in HamF-12 media containing 18% FBS, the cells were cultured for 67 hours in HamF-12 media containing 10% FBS with various concentrations of HN polypeptides; and cell death was measured by trypan blue exclusion assay. FALS-associated mutant cDNAs of SOD1 were also similarly transfected, and examined for their neurotoxicity effect.

[0036]

To obtain the culture supernatant of F11 cells transfected with pHN (CM/F11-pHN), F11 cells were transfected with pHN in the absence of serum by lipofection for 3 hours (1 μg of pHN, 2 μl of LipofectAMINE, 4 μk of Plus reagent); and were cultured for 2 hours in HamF-12 media containing 18% FBS. Thereafter, the media was exchanged with HamF-12 media containing 10% FBS; and the cells were additionally cultured for 67 hours. CM/F11-pHN was obtained by freeze-thawing the culture media once. For immunoblot analysis of CM/F11-pHN, CM/F11-pHNG, and CM/F11-pHNA, protease inhibitor cocktail (Boehringer Mannheim, Cat. #1697498; one tablet was dissolved in 2 ml of distilled water, and a volume 1/25 to that of the sample was added to the sample) was added to the culture media that had not been freeze-thawed. Tris/Tricine gel electrophoresis was performed according to the litearture (Schagger, H. and von Jagow, G. (1987) Analytical Biochemistry 166, 168-179).

[0037]

F11/EcR/V642I cells were established using ecdysone-inducible V642I APP expression plasmid. First, the co-expression vector pVgRXR was transfected into F11 cells (Invitrogen) and cells were subjected to Zeocin selection to establish F11 cells (F11/EcR cells) that stably overexpress both ecdysone receptor EcR and the retinoid X receptor RXR. V642I APP cDNA was inserted into pIND vector (Invitrogen), having multiple copies of ecdysone responsive sequences; and after transfection of the vector into F11/EcR cells, G418 selection was performed. F11/EcR/V642I cells were cloned by limiting dilution. F11/EcR/V642I cells were cultured in HamF-12 media containing 18%

FBS and antibiotics. Before ecdysone treatment, the cells were cultured for 24 hours in the presence of 10% FBS. Then, the cell was treated with ecdysone (40 μM Ponasteron; Invitrogen Cat. #H101-01) for 48 to 72 hours in the presence of 10% FBS. Cell death occurred to each F11/EcR/V642I cell, in response to ecdysone treatment; and the cell mortality 72 hours after treatment in all cells was 60 to 70%, which reached 80 to 90% after 96 hours from the treatment. A more detailed analysis of F11/EcR/V642I cells is described elsewhere (see International Application No. PCT/JP99/04771).

[0038]

F11/EcR cell experiment using ecdysone was conducted as follows: F11/EcR cells were seeded at 7×10^4/well into a 6-well plate; cultured for 12 to 16 hours in HamF-12 media containing 18% FBS; and were similarly transfected in the absence of serum for 3 hours with 1 μg of ecdysone-inducible ~~plasmid~~ alone, or with 1 μg of HN-encoding plasmid, as mentioned above. After culturing for 12 to 16 hours in HamF-12 media containing 18% FBS, the cells were cultured for 2 hours in HamF-12 media containing 10% FBS, then ecdysone (Ponasterone) was added to the media (final concentration of 40 μM). Cell death was measured 72 hours after ecdysone treatment. Experiments using synthetic HN polypeptides were conducted as follows: cells were similarly transfected for 3 hours with FAD genes in the absence of serum; cultured for 12 to 16 hours in HamF-12 media containing 18% FBS; cultured for 2 hours in HamF-12 media containing 10% FBS and various concentrations of HN polypeptide; and then, 40 μM Ponasterone was added to the media. Cell death was measured by trypan blue exclusion assay after 72 hours from the ecdysone treatment. HD/SCA-associated Q79 cDNAs were also similarly transfected, and the neurotoxicity effect was tested.

[0039]

Primary culture of mouse cortical neurons was performed in poly-D-lysine-coated 24-well plates (Sumitomo Bakelite) as described in literature (Eksioglu, Y. Z. et al. (1994) Brain Res. 644, 282-90). The prepared neurons (1.25×10^5/well, 250 μl media/well) were preincubated in the absence or presence of 10 nM or 10 μM sHN polypeptides for 16 hours; and were treated with 25 μM Aβ1-43 in the

absence or presence of sHN polypeptides at the same concentrations for 24 to 72 hours. Since primary cultured neurons are damaged even by temporary dryness during medium exchange, treatment of the cells by Aβ1-43 was performed as follows. First, half of the volume of the old medium (125 μl) was discarded. Then, 125 μl of pre-warmed fresh medium containing 50 μM Aβ1-43 and sHN with a volume indicated above were added to the culture.

[0040]

Trypan blue exclusion assay was performed as follows. Without prewashing, the cells were suspended with gentle pipetting into a serum-free media. 50 μl of 0.4% trypan blue solution (Sigma, Cat. #T-8154) were added (final concentration of 0.08%) to 200 μl cell suspension, and the suspension was mixed at room temperature. Within 3 minutes of the trypan blue solution addition, stained cells were counted. Cell mortality was determined [100-cell survival rate (%)] based on the stained cell count. LDH assay was performed using a kit (LDH-Cytotoxic Test; Wako Pure Chemical Industries, Cat. #299-50601) by sampling 6 μl of media in which neurons were cultured. Calcein staining was performed as described in literature (Bozyczko-Coyne, D. et al. (1993) Journal of Neuroscience Methods 50, 205-216). Specifically, 6 μM Calcein-AM {3',6'-Di-(O-acetyl)-2',7'-bis[N,N-bis(carboxymethyl)aminomethyl]fluorescein, tetraacetoxymethyl ester; Dojindo, Cat. #349-07201} was added to the neurons; and 30 minutes or longer after Calcein-AM treatment, fluorescence (ex = 490 nm, em = 515 nm) was measured by fluorescence microscopy.

All experiments described herein was performed at least three times by repeating independent transfection or treatment. Student's t test was performed as the statistical analysis.

[0041]

[Example 1] <u>Identification of Humanin</u>

The F11 cell, established by fusing E17.5 rat primary cultured neurons and mouse neuroblastoma NTG18, is an immortalized cell model of primary cultured neurons (Platika, D. et al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 3499-3503). Without a differentiation stimulus,

the cell maintains typical characteristics of primary cultured neurons, such as the production of an activation potential (Platika, D. et al. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 3499-3503). The present inventor discovered that upon transfection of F11 cells with cDNA encoding V642I/F/G APP, i.e. three kinds of FAD causative genes, transient expression of V642 mutant APP causes cell death (Yamatsuji, T. et al. (1996) Science 272, 1349-1352). Accordingly, the present inventor used the recently developed ecdysone-inducible system (No, D. et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 3346-51) to construct F11 clones wherein the V642I APP is inducible. F11 cells wherein the expression of V642I APP can be induced were established as follows: first, F11 clones (F11/EcR) that overexpress both ecdysone receptor and RXR were established; and then, the cells were stably transfected with pIND-V642I APP, which encodes V642I APP cDNA that is expressed by an HSV promoter placed under the control of ecdysone responsive sequences. In their original form, F11/EcR/V642I clone cells established as above hardly expresses V642I APP. However, overexpression of V642I APP from the cells due to ecdysone treatment was confirmed. Furthermore, in response to ecdysone treatment, cell death was induced in all of the F11/EcR/V642I cells; and cell mortality in all F11/EcR/V642I cells reached 60 to 70% after 72 hours from the treatment, and 80 to 90% after 96 hours from the treatment.

[0042]

Using these cells, and basically following the method developed by D’Adamio et al. (D'Adamio, L. et al. (1997) Semin. Immunol. 9, 17-23), “death trap screening” was performed using a modified version of the method of D’Adamio et al. First, F11/EcR/V642I cells were transfected with mammalian expression cDNA library [cDNA was prepared from brain samples of Alzheimer patients; and the library was constructed using mammalian cell expression vector pEF-BOS, having the elongation factor promoter] (Mizushima and Nagata, 1990, Nucleic Acids Res. 18: 5322); the cells were treated with ecdysone for 72 hours; and plasmids were collected from surviving cells. The procedure was repeated 3 times, and ultimately, plasmids of about 250 clones were obtained. The clones were categorized into 36 groups that cross hybridize to each other by dot blot hybridization using

respective plasmids. The largest group comprised 28 clones. Focusing on this group of cDNAs, the present inventor sequenced all the clones. As a result, clones belonging to this group generally consisted of a cDNA having a fused sequence of 1535 bp; specifically, 5′ sequence homologous to the non-coding region of Wnt-13, a 3′ sequence homologous to the mitochondrial 16S ribosomal RNA, and a poly(A) region at the C-terminus (Fig. 1). After sequencing each clone, whether the co-transfection of respective clones significantly suppresses cell death in F11 cells transfected with V642I APP cDNA was assayed. As a result of comparing the sequences which demonstrated a cell death suppression activity, an rescuing activity against cell death induced by V642I APP was found to be encoded by a 75 bp open reading frame (ORF) (5′-ATGGCTCCACGAGGGTTCAGCTGTCTCTTACTTTTAACCAGTGAAATTGACCTGCCCGTG AAGAGGCGGGCATGA-3′ /SEQ ID NO: 3) encoding a novel 24-amino acid polypeptide "MAPRGFSCLLLLTSEIDLPVKRRA" (SEQ ID NO: 4). The present inventor dubbed the molecule Humanin (HN).

[0043]

[Example 2] Suppressive effect of respective clones on cell death induced by AD genes

Fig. 2 to 4 demonstrate the effects of co-transfection of respective clones belonging to this group. When F11/EcR cells (F11 clone stably expressing EcR and RXR, wherein the expression of genes encoded by pIND plasmid are induced by ecdysone) were transiently transfected with pIND encoding V642I APP in the absence of ecdysone (non-V642I APP inducing conditions), 72 hours later, cell death occurred in about 20% of the cells, whereas cell death occurred in a significantly high proportion (50 to 60%) of cells in the presence of ecdysone (V642I APP inducing conditions)(Fig. 2). F11/EcR cells transfected with DT63-encoding pEF-BOS, in addition to V642I APP-encoding pIND, demonstrated no significant increase of cell death induced by ecdysone even in the presence of ecdysone. On the other hand, cells transfected with pEF-BOS or pEF-BOS encoding DT171, demonstrated significant increase in cell death in response to ecdysone. Fig. 3 depicts an experimental result in transient

transfection system of pcDNAs encoding respective AD genes, demonstrating the effect of DT63 on neuronal death induced by the four FAD genes (V642I APP, NL APP, M146L PS-1, and N141I PS-2), respectively. When F11 cells were co-transfected with empty pEF-BOS in addition to the pcDNA encoding V642I APP, NL APP, M146L PS-1, or N141I PS-2, incubation for 72 hours lead to cell death in 50 to 70% of the cells. The transfection efficiency under this condition was about 60 to 70%, which indicates that cell death occurred after 72 hours from transfection in most of the cells expressing one of the FAD genes. Increase in cell death was dramatically suppressed by transfecting F11 cells with DT63-encoding pEF-BOS in addition to one of the FAD genes. This indicates that, DT63 antagonizes all of the cell deaths induced by the four AD genes with a high efficiency. Fig. 4 demonstrates the effect of other DT clones that contain the entire Humanin sequence, and other DT clones that do not contain the entire sequence (DT29, DT44, and DT171). Although marked suppression of cell death induced by V642I APP was demonstrated with DT29 and DT44, which are clones encoding the entire Humanin sequence, action of antagonizing cell death induced by V642I APP could not be confirmed with DT171 lacking the first ATG codon of Humanin. These data indicate that the ORF encoded by Humanin protects neurons from cell death caused by all four AD genes.

[0044]

[Example 3] <u>Suppression effect of Humanin on cell death induced by AD genes</u>

Therefore, the present inventor subcloned Humanin cDNA into the pFLAG vector (pHN), and directly investigated the effect of pHN towards neuronal death caused by each of V642I APP, NL-APP, M146L PS-1, and N141I PS-2. As expected, transfection of pHN into F11 cells hardly showed toxicity, and furthermore, detoxified the toxicity by the AD genes (Fig. 5). The antagonizing activity is not the result of suppression of the expression of respective AD genes by pHN. This is verified by the fact that co-transfected pHN doesn't change the expression of FAD genes expressed from the CMV promoter, which was indicated from the finding that pHN co-transfection didn't change

the expression of EGFP expressed from the CMV promoter (data not shown. Furthermore, immunoblotting of V642I APP, NL-APP, and N141I PS-2 confirmed that co-transfection of pHN hardly has any effect on the expression of these genes (data not shown). In the course of experiments, the culture supernatant of F11 cells transfected with pHN (CM/F11-pHN) was demonstrated to significantly suppress cell death induced by V642I APP. Under the presence of CM/F11-pHN, cell death decreased significantly in F11 cells transfected with V642I APP, compared with F11 cells transfected with V642I APP under the absence of CM/F11-pHN (Fig. 6). The result suggests that polypeptides generated through transcription from pHN are secreted in CM/F11-pHN. Fig. 7 demonstrates the result of investigation on immunoreactivity of HN in CM/F11-pHN using anti-FLAG antibodies. CM/F11-pHN showed a single band at 3 to 4 kDa, indicating immunoreactivity of HN, ~~—~~the size of wishich concordant with the expected molecular weight for FLAG-fused HN (3837 Da). Semiquantitative analysis using synthetic FLAG-fused HN polypeptide (MAPRGFSCLLLLTSEIDLPVKRRAGT<u>DYKDDDDK</u>: Flag tag is underlined) (SEQ ID NO: 5) demonstrated that HN is induced in CM/F11-pHN at a concentration of 8 to 9 μM. These findings indicate that HN is transcribed from pHN and is secreted into the culture supernatant.

[0045]

[Example 4] <u>Suppressive effect of synthetic HN polypeptide on cell death induced by V642I APP</u>

Next, the present inventor synthesized a synthetic HN polypeptide MAPRGFSCLLLLTSEIDLPVKRRA (SEQ ID NO: 4), and investigated its action on neuronal death induced by V642I APP by adding the polypeptide extracellularly. Cell death induced by V642I APP was dramatically suppressed by transfecting F11 cells with V642I APP cDNA and culturing the cells in the presence of 10 μM synthetic HN polypeptide (sHN) (Fig. 8). Only an extremely weak suppression was indicated at 10 nM sHN. The suppressive action was dependent on the concentration of sHN addded, and at the level of 1 to 10 μM polypeptide, complete suppression could be achieved. IC_{50} value was about 100 nM. The dose-dependent curve agrees with the fact that HN secreted at

a level of about 10 μM into CM/F11-pHN effectively suppressed cell death induced by V642I APP. By taking into account the fact that secreted HN was accumulated gradually up to 10 μM for 72 hours, it can be explained that cell death induced by V642I APP tends not to be completely suppressed by CM/F11pHN.

[0046]

[Example 5] Suppressive effect of structural derivatives of Humanin polypeptide on cell death induced by V642I APP

The present inventor further examined whether the cell death suppressive action of sHN is dependent on the specific primary structure. A complete antagonizing effect on cell death induced by V642I APP could be observed at a concentration of 10 nM or less with S14G (MAPRGFSCLLLLTGEIDLPVKRRA: the underlined G replaces S; called HNG) (SEQ ID NO: 6) as the polypeptide, and IC_{50} of the polypeptide was about 100 pM. In contrast, C8A HN polypeptide (MAPRGFSALLLLTSEIDLPVKRRA: underlined A replaces C; called HNA) (SEQ ID NO: 7) did not significantly suppress cell death induced by V642I APP at concentrations up to 100 μM. The importance of Cys at position 8 was also suggested from the result obtained using an HN dimer (C8-C8 HN), bound through Cys at position 8. The antagonizing action level of C8-C8 HN was in between those of the original HN and HNA. On the contrary, a derivative wherein the HN C-terminal KRRA was substituted with AAAA indicated similar functional activity to the original HN polypeptide. These results indicate that the primary structure has a fundamental role in the rescue activity of Humanin, and that particular amino acid residues have a predetermined role.

[0047]

[Example 6] Suppressive effect of Humanin polypeptides and structural derivatives thereof on cell death induced by AD genes

Next, the effect of synthetic HNG (sHNG) and synthetic HNA (sHNA) on cell death induced by other AD genes, more specifically, those induced by NL-APP, M146L PS-1, and N141I PS-2 was investigated. As indicated in Fig. 9, the original sHN demonstrated similar dose-responsiveness on cell death induced by any of the AD genes,

and blocked neuronal death induced by the AD genes at a concentration of 1 μM. Up to a concentration of 100 μM, sHNA did not antagonize cell death by any of the AD genes. In contrast, sHNG completely suppressed cell death caused by any of the FAD genes at a concentration of 10 nM or less. This indicates that the action of Humanin is enhanced 100 to 1000 fold by S14G substitution. Taking the action of sHNG on cell death induced by V642I APP togeter, sHNG at a concentration of 10 nM or less, completely antagonizes neuronal death induced by all of the four different types of FAD-linked genes.

[0048]

[Example 7] Cell death suppressive effect by the transfection of vectors expressing Humanin and structural derivative thereof

Next, cell death suppressive effect of HNG or HNA-encoding plasmid (pHNG or pHNA, respectively) compared to pHN was investigated to confirm the data obtained from synthetic polypeptides. As demonstrated in Fig. 10, similarly to the case with pHN, co-transfection of pHNG completely suppressed cell death induced by all of the 4 types of AD genes. In contrast, even though HNA polypeptides were produced and secreted into the media by pHNA co-transfection, similarly to the cases with pHN, suppression of cell death caused by any of the AD genes couldn't be observed (Fig. 7). These data obtained from each of the plasmids encoding HN not only support the summary of the previously mentioned HN polypeptide structural dependency, but also suggests that HNG concentration within CM/F11-pHNG (culture supernatant of F11 cells transfected with pHNG) exceeds 10 nM. Concordant with the result, according to the immunoblot analysis, the culture supernatant of F11 cells transfected with pHNG (CM/F11-pHNG) contained about 10 μM of HNG polypeptide (Fig. 7). These data indicate that the cell death suppression activity of Humanin is determined by its specific amino acid structure, and that the rescue action caused by extracellularly adding HN polypeptides may be also caused by intracellularly expressing HN cDNA.

[0049]

[Example 8] Specificity of the cell death suppressive effect of

Humanin

To elucidate the specificity of Humanin action, the ability of HN cDNA or HN polypeptide to antagonize cell death induced by causative genes of other neurodegenerative diseases was investigated. Polyglutamine Q79, having 72 repeats, is considered to be the cause of Huntington's disease (HD) and certain types of spinocerebellar ataxia (SCA) (Kakizuka, A. (1997) Curr. Opin. Neurol. 10, 285-90). In accordance with the report that Q79 expression causes neuronal death (Ikeda, H. et al. (1996) Nat. Genet. 13, 196-202), F11 cells underwent cell death due to the expression of Q79. The present inventor used ecdysone inducing system in order to minimize the effect of pHN transfection or sHN treatment on Q79 expression. The ratio of cell death was examined in the presence or absence of ecdysone by transfecting F11/EcR cells with Q79 plasmid, the expression of which is induced by ecdysone (pDN-E/G5H-Q79). In this system, cell mortality markedly increased in response to ecdysone treatment when F11/EcR cells were transfected with pDN-E/G5H-Q79 together with pFLAG (Fig. 11A). Similarly, high proportions of cell death of F11/EcR cells, transfected with pDN-E/G5H-Q79 together with pHN, pHNG, or pHNA, were induced by ecdysone treatment. Further, F11/EcR cell death caused by ecdysone-induced expression of V642I APP or NL-APP was effectively suppressed by the co-transfection of pHN (Fig. 11B). Cell death induced by Q79 was not suppressed in the experiment using sHN (Fig. 11C). Extensive cell death was caused by ecdysone when F11/EcR cells were transfected with pDN-E/G5H-Q79, even in the presence of sHN, sHNG, or sHNA at a concentration of sHN or sHNG that enables complete suppression of F11/EcR cell death caused by any one of the 4 types of FAD genes, just as in the absence of sHN, sHNG, or sHNA.

[0050]

Additionally, the present inventor investigated the effect of Humanin on neuronal death induced by mutants of Cu/Zn-dependent superoxide dismutase (SOD1), i.e. A4T, G85R, or G93A, associated with familial amyotrophic lateral sclerosis (FALS). In accordance with previous reports reporting that expression of FALS-associated SOD1 mutants cause cell death of mammalian neurons (Rabizadeh, S. et al.

(1995) Proc. Natl. Acad. Sci. U.S.A. 92, 3024-8; Ghadge, G. D. et al. (1997) J. Neurosci. 17, 8756-66), significant cell death was induced with all of the mutants by transfecting F11 cells with a cDNA that expresses one of the mutants. Further, a similar high cell mortality was induced when F11 cells were co-transfected with pHN in addition to each SOD1 mutant gene (Fig. 12A). As demonstrated in Fig. 12B, cell death caused by one of the FALS-associated SOD1 mutants couldn't be suppressed significantly with 100 μM of any of sHN, sHNG, or sHNA. These data suggest that Humanin activates the intracellular mechanism for invalidating the cell death execution mechanism triggered by the FAD genes, but does not function on cell death caused by HD/SCA or FALS genes, and verify that the antagonizing effects of Humanin cDNA and Humanin polypeptides are common and specific to neuronal death associated with AD.

[0051]

[Example 9] <u>Suppressive effect of Humanin on cell death of primary neuronal culture</u>

The present inventor examined the protection of primary cultured neurons by Humanin from damages associated with AD. Aβ is the major peptide component of senile plaque and an extracellular deposit that pathologically characterizes an AD brain, and is suggested to be associated with the pathological mechanism of AD (Selkoe, D. J. (1994) J. Neuropathol. Exp. Neurol. 53, 438-47; Cummings, J. L. et al. (1998) Neurology 51, S2-17; discussion S65-67). Aβ treatment has been reported to induce cell death of primary cultured neurons (Loo, D. T. et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7951-7955). As demonstrated in Fig. 13, extensive cell death accompanied by dystrophic neuritic changes of the axon was induced in primary cultured cortical neurons treated with 25 μM Aβ1-43 for 48 to 72 hours in the presence or absence of N2 supplement (Fig. 13 and 14; Fig 13a for culture in the absence of N2 supplement). Cell death induced by Aβ, as well as dystrophic neuritic changes of the axon were dramatically suppressed in primary cultured neurons pre-treated with 10 μM sHN. Cell death (measured by trypan blue exclusion) and cell damage (measured by LDH released from the cells)

was increased by aβ1-43. These indices of cell survival (i.e., cell death and cell damage) were restored to a level observed under basal conditions by the treatment with 10 μM or more sHN. Under the same conditions, 100 ng/ml of NGF did not show effects to rescue cell death of neurons induced by Aβ (data not shown). In spite of the fact that sHN demonstrated a dramatic effect in antagonizing neuronal death induced by Aβ, similar treatment of neurons with 10 μM sHN could not prevent the toxic effect of 20 μM etoposide on primary cultured neurons (data not shown). Etoposide is an anticancer agent and has been reported to induce cell death of primary cultured neurons (Nakajima, M. et al. (1994) Brain Res. 641, 350-2). These findings support the idea that Humanin rescue neuronal death induced by Aβ1-43 by a selective mechanism. As indicated in the experiment of AD genes using F11 cells, 10 nM sHNG almost completely protected cells from cell death and dystrophic neuritic changes of the axon caused by Aβ1-43, but 10 nM sHN or 10 μM sHNA both did not show any effect against toxicity of Aβ (morphological change is shown in Fig. 15). The result was confirmed by the measurement of viable cells with Calcein staining assay (Fig. 16) and by the measurement of death cells with trypan blue exclusion assay (Fig. 17). These data demonstrated that Humanin was shown to have similar effects as anti-AD factor on primary cultured neurons as well as on cloned neurons. Furthermore, these data suggest the existence of a receptor (group of receptors) that specifically recognizes the Humanin structure, common between F11 cells and primary cultured neurons.

[0052]

[Effects of the Invention]

The present invention provides Humanin polypeptide, which has the ability to antagonize neurodegeneration associated with Alzheimer's disease. The polypeptide is the first molecule which has the ability to antagonize cell death of neurons induced by four kinds of AD genes and Aβ. Further, the polypeptide can antagonize cell death of cloned neurons induced by V642I APP, NL-APP, PS-1 mutants, and PS-2 mutants, and cell death of primary cultured neurons induced by Aβ1-43. Rescue factors acting extracellularly and possessing a wide

spectrum, antagonizing Aβ and all of the known types of FAD genes, had never been characterized before. Usefulness of HNG for elucidating the mechanism of neuronal death associated with Alzheimer's disease, as well as the extreme usefulness of HNG for clinical applications was particularly demonstrated by the fact that neurotoxicity associated with AD is completely antagonized with 10 nM or less of HNG. The protective action of HNG is extremely high, and HNG has generality and stringent specificity. The novel polypeptide, Humanin, and its structural derivatives that have capability equivalent to the polypeptide or more are extremely useful as pharmaceuticals to prevent neuronal death associated with Alzheimer's disease, and as seed compounds for the development of new pharmaceuticals for Alzheimer's disease.

[0053]
[Sequence Listing]

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<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: HNG, S14G substitution of Humanin

<400> 6
Met Ala Pro Arg Gly Phe Ser Cys Leu Leu Leu Leu Thr Gly Glu Ile
1 5 10 15

Asp Leu Pro Val Lys Arg Arg Ala
20

<210> 7
<211> 24
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: HNA, C8A substitution of Humanin

<400> 7
Met Ala Pro Arg Gly Phe Ser Ala Leu Leu Leu Leu Thr Ser Glu Ile
1 5 10 15

Asp Leu Pro Val Lys Arg Arg Ala

[Brief Description of the Drawings]

[Fig. 1]

Fig. 1 depicts a schematic illustration of the region in Humanin cDNA clone that encodes a polypeptide that antagonizes cell death caused by V642I APP.

DNA fragments were aligned with respect to the longest sequence (from -934 to 592; the number 1 nucleotide corresponds to the first nucleotide of Humanin ORF, and the nucleotide adjacent to it is numbered -1). Activities of the fragments against F11/EcR cell death induced by V642I APP are indicated under the item "rescue activity". F11/EcR cells were transfected for 3 hours with pIND (1 μg), encoding V642I APP, and 1 μg of either pEF-BOS or pEF-BOS encoding each of the DNA fragments; and then, were treated with ecdysone for 72 hours. Cell death was measured by trypan blue exclusion assay. A DNA fragment was determined to antagonize cell death (described as "Y" under the item "rescue activity") when the mortality of cells transfected with the DNA fragment showed a statistically significant difference with that of cells transfected with pEF-BOS. "N" indicates the absence of such a significant antagonizing activity.

[Fig. 2]

Fig. 2 depicts a graph demonstrating the effects of DT63 clone and DT171 clone on neuronal death caused by ecdysone-induced expression of V642I APP. F11/EcR cells were transfected with ecdysone-inducible V642I APP plasmid, and any one of pEF-BOS, DT63, or DT171 (DT63 and DT171 were cloned in pEF-BOS), and were treated with Ponasterone (ecdysone). A group without ecdysone treatment was also set up. 72 hours after ecdysone treatment, cell death was measured by trypan blue exclusion assay. Cell death of the group without ecdysone treatment was measured similarly. The values and error bars in the graph represent mean ±S.D. values of three independent transfection/treatment experiments. DT63 and DT171 are shown in Fig. 1.

[Fig. 3]

Figure 3 depicts a graph demonstrating the effect of DT63 clone on neuronal death induced by the expression of the FAD gene. F11 cells were transfected with pcDNA; or pcDNA encoding V642I APP, NL-APP, M146L PS-1, or N141I PS-2; and pEF-BOS(vec); or pEF-BOS encoding DT63; and were cultured for 72 hours. Cell death was measured by trypan blue exclusion assay.

[Fig. 4]

Fig. 4 depicts graphs indicating the effects of DT29, DT44, and DT171 clones on F11 cell death caused by FAD gene transfection. Similarly to Figure 3, F11 cells were transfected with pcDNA; or pcDNA encoding either V642I APP, NL-APP, M146L PS-1, or N141I PS-2; and pEF-BOS(pBOS); or pEF-BOS encoding DT clone; and were cultured for 72 hours. Cell death was measured by trypan blue exclusion assay. DT29 and DT44 are shown in Figure 1. Basal cell mortality (no transfection, pcDNA+ pBOS) was concordant in the three experiments performed simultaneously. Similar experiments were performed at least three times.

[Fig. 5]

Fig. 5 depicts graphs demonstrating the effect of Humanin-encoding plasmid pHN on neuronal death induced by expression of a FAD gene. F11 cells were transfected with an empty vector (pcDNA); or pcDNA encoding V642I APP, NL-APP, M146L PS-1, or N141I PS-2; and pFLAG; or pFLAG encoding HN (pHN); and were cultured for 72 hours. Cell death was measured by trypan blue exclusion assay.

[Fig. 6]

Fig. 6 depicts a graph demonstrating the suppressive effect of a culture supernatant from pHN-transfected F11 cells on neuronal death induced by V642I APP. F11 cells were transfected for 3 hours with either pcDNA or pcDNA encoding V642I APP, in the absence of serum; cultured in HamF-12 containing 18% FBS for 2 hours; and cultured in CM/F11-pHN or fresh media (fresh HamF-12 containing 18% FBS) for 67 hours. 72 hours after the transfection, cell death was measured by

trypan blue exclusion assay. $p < 0.01$; according to a Student's t test, "n.s." represents no significant difference.

[Fig. 7]

Fig. 7 depicts drawings demonstrating the immunoreactivity of Humanin polypeptides contained in the culture supernatant of F11 cells transfected with pHN, pHNG, or pHNA. The cocktail of protease inhibitors was added to the culture supernatatnt and the mixed solution was subjected to Tris/Tricine gel electrophoresis and then to immunoblotting with anti-FLAG antibody. The 3 lanes on the right demonstrate the results of immunoblotting on sHN-FLAG (sHN to which DYKDDDDK is bound at C-terminus) (SEQ ID NO: 6) with indicated concentrations, to conduct the semiquantitative analysis of HN polypeptides contained in the culture supernatant.

[Fig. 8]

Fig. 8 depicts graphs demonstrating the effect of synthetic HN (sHN) and structural derivatives thereof on neuronal death induced by V642I APP. F11 cells were transfected with pcDNA encoding V642I APP; and were treated with various concentrations of sHN (Authentic Humanin), sHNG (S14G), sHNA (C8A), dimer form of sHN through C8 (C8-C8), and sHN in which the C-terminal KRRA was replaced with AAAA (KRRA21/22/23/24AAAA). 72 hours after the transfection, cell death was measured by trypan blue exclusion assay.

[Fig. 9]

Fig. 9 depicts graphs demonstrating the effect of synthetic HN and its structural derivatives (sHN, sHNG, or sHNA) on neuronal death induced by other AD genes (M146L PS-1, N141I PS-2, or NL-APP). Similarly to Fig. 8, F11 cells were transfected with M146L PS-1, N141I PS-2, or NL-APP cDNA; and were treated with various concentrations of sHN (Authentic Humanin), sHNG (S14G), or sHNA (C8A). 72 hours after the transfection, cell death was measured by trypan blue exclusion assay.

[Fig. 10]

Fig. 10 depicts bar graphs demonstrating the effect of pHN, pHNG, or pHNA on neuronal death induced by the expression of FAD genes. F11 cells were transfected with empty vector (pcDNA) or pcDNA encoding V642I APP, NL-APP, M146L PS-1, or N141I PS-2, and pFLAG or pFLAG encoding HN (pHN, pHNG, or pHNA); and were cultured for 72 hours. Cell death was measured by trypan blue exclusion assay.

[Fig.11]

Fig. 11 depicts graphs demonstrating the lack of effect of HN and structural derivatives thereof on neuronal death induced by polyglutamine repeat Q79.

Panel A: demonstrates the lack of the effect of pHN, pHNG, or pHNA on neuronal death caused by Q79 induced by ecdysone. F11/EcR cells were transfected with ecdysone-inducible type Q79 expression vector, and empty vector (pFLAG), pHN, pHNG, or pHNA, and were cultured for 72 hours in the presence or absence of ecdysone. Cell death was measured by trypan blue exclusion assay.

Panel B: demonstrates a significant suppressive effect by pHN co-transfection on neuronal death caused by ecdysone-induced expression of NL-APP or V642I APP. Under the same conditions as in Panel A, F11/EcR cells were transfected with ecdysone-inducible NL-APP or V642I APP plasmid, and pFLAG or pHN, and then, were cultured for 72 hours in the presence or absence of ecdysone. Cell death was measured by trypan blue exclusion assay.

Panel C: demonstrates the lack of effects of sHN, sHNG, or sHNA on neuronal death induced by ecdysone-inducible expression of Q79. F11/EcR cells were transfected with ecdysone-inducible Q79 plasmids; treated with 1 μM sHN, sHNG, or sHNA; and then, were treated with ecdysone. 72 hours after the initiation of the exdysone treatment, cell death was measured by trypan blue exclusion assay.

[Fig. 12]

Fig. 12 depicts graphs demonstrating the lack of the effect of HN and structural derivatives thereof on neuronal death induced by the FALS-associated SOD1 mutants.

Panel A: demonstrates the lack of the effect of pHN

co-transfection on neuronal death induced by the expression of the FALS-related SOD1 mutants. F11 cells were transfected with pEF-BOS encoding the FALS-associated mutant SOD1 (A4T, G85R, or G93A mutants of SOD1) and empty vector (pFLAG) or pHN. Cell death was measured by trypan blue exclusion assay.

Panel B: demonstrates the lack of the effect of sHN, sHNG, or sHNA on neuronal death induced by the expression of the FALS-associated SOD1 mutants. F11 cells were transfected with pEF-BOS encoding A4T, G85R, or G93A SOD1, and were treated with 100 μM sHN, sHNG, or sHNA. Cell death was then measured by trypan blue exclusion assay.

[Fig. 13]

Fig. 13 depicts a drawing demonstrating the effect of HN polypeptide (sHN) on Aβ1-43-induced cell death of primary cultured neurons. Primary cultured cortical neurons were treated for 72 hours with 25 μM Aβ1-43 in the presence or absence of sHN. "Aβ1-43 + 10 μM sHN", one addition of 10 μM sHN 16 hours before Aβ1-43 treatment; "Aβ1-43 + >10 μM sHN", four additions of 10 μM sHN (first: 16 hrs before Aβ1-43 treatment; second: at the Aβ1-43 treatment; third: 24 hrs after Aβ1-43 treatment; fourth: 48 hrs after Aβ1-43 treatment).

72 hrs after Aβ treatment, cell death was measured by trypan-blue exclusion assay. Cytotoxicity was monitored by LDH release to culture media. The experiments were conducted in the presence (+) or absence (-) of N2 supplement. Each experiment was conducted using two neuronal cultures and the means were represented. Similar experiments were conducted at least three times. S.E. was less than 5% to its corresponding mean value.

[Fig. 14]

Fig. 14 depicts phase-contrast microgram representing the effect of sHN on Aβ1-43-induced cell death of primary cultured neurons. Primary cultured cortical neurons were treated with 25 μM of Aβ1-43 for 72 hrs in the presence (> 10 μM) (four treatments with 10 μM sHN in the same manner as Fig. 13) or absence of sHN. Representative micrograph was shown.

[Fig. 15]

Fig. 15 depicts phase-contrast microgram representing the effect of sHN, sHNG, or sHNA on Aβ1-43-induced cell death of primary cultured neurons. Representative micrographs were shown. Primary cultured cortical neurons were treated with 25 μM of Aβ1-43 for 72 hrs under the presence of N2 supplement in the presence or absence of sHN (10 nM, 10 μM), 10 nM sHNG, or 10 μM sHNA. In these experiments, HN polypeptides at indicated final concentration were added once 16hrs before Aβ1-43 treatment. Similar experiments were conducted at least three times and reproducible results were obtained.

[Fig. 16]

Fig. 16 depicts fluorescent microgram representing the effect of sHN, sHNG, or sHNA on Aβ1-43-induced cell death of primary cultured neurons examined by Calcein-AM staining. Representative micrographs were shown. Primary cultured cortical neurons were treated with 25 μM of Aβ1-43 for 72 hrs under the presence of N2 supplement in the presence or absence of sHN (10 nM, 10 μM), 10 nM sHNG, or 10 μM sHNA. In these experiments, HN polypeptides at indicated final concentration were added once 16hrs before Aβ1-43 treatment. Similar experiments were conducted at least three times and reproducible results were obtained. Calcein-AM staining was conducted 72 hrs after Aβ1-43 treatment. The fluorescence in cytoplasm represents that the cell is viable.

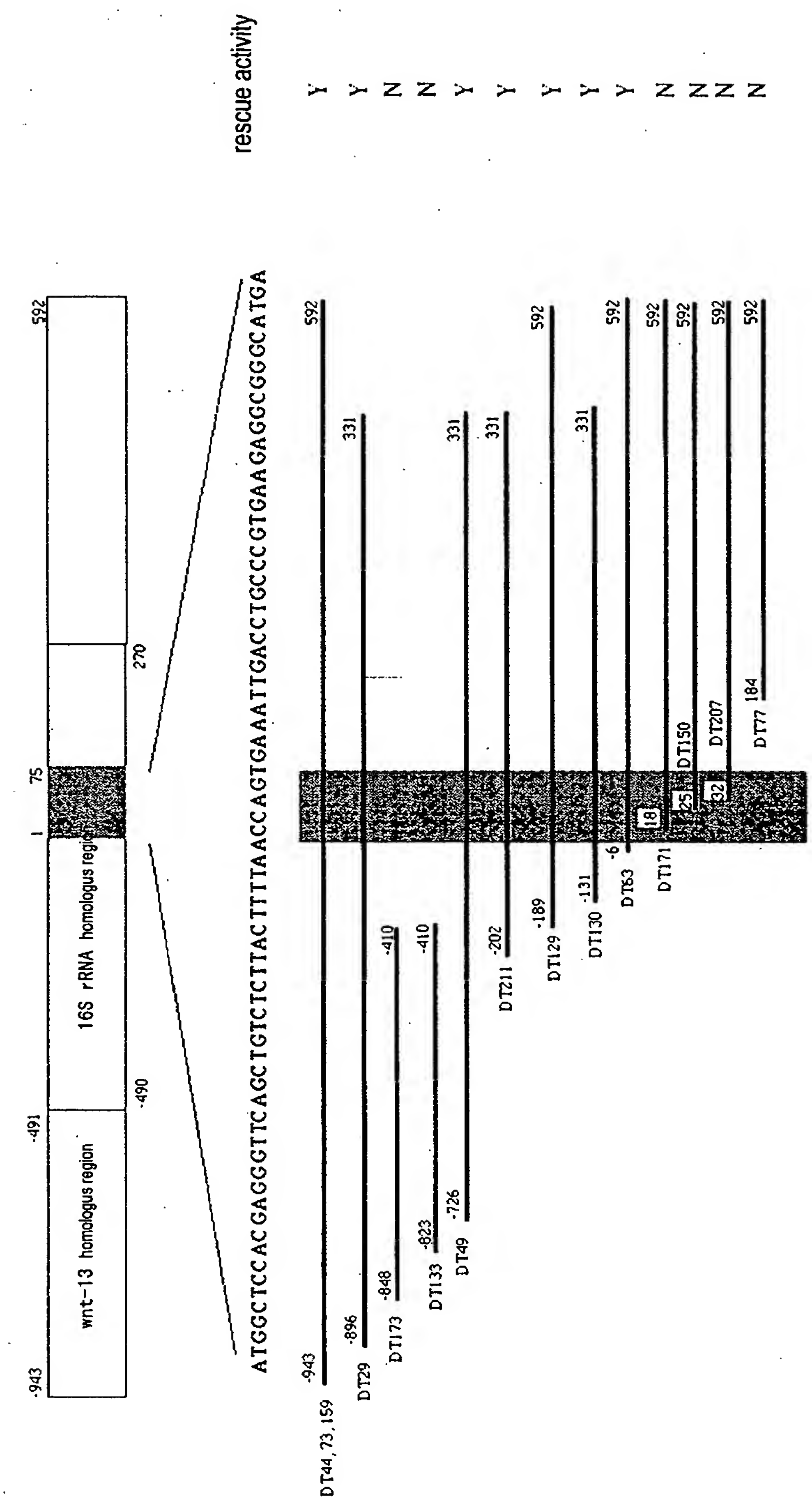
[Fig. 17]

Fig. 17 depicts a drawing representing the effect of sHN, sHNG, or sHNA on Aβ1-43-induced cell death of primary cultured neurons. Primary cultured cortical neurons were treated with 25 μM of Aβ1-43 for 72 hrs under the presence of N2 supplement in the presence or absence of sHN (10 nM, 10 μM), 10 nM sHNG, or 10 μM sHNA. Cell death was measured 72 hrs after Aβ1-43 treatment by trypan-blue exclusion assay. In these experiments, HN polypeptides at indicated final concentration were added once 16hrs before Aβ1-43 treatment. Similar experiments were conducted at least three times and reproducible

results were obtained.

[Document Name] Drawings

[Figure 1]


rescue activity
-943
-491
wnt-13 homologus region
-490
16S rRNA homologus region
1
75
270
592
ATGGCTCCACGAGGGTTCAGCTGTCTCTTACTTTTAACCAGTGAAATTGACCTGCCCGTGAAGAGGCGGCATGA
DT44, 73, 159
-943
592
Y
DT29
-896
331
Y
DT173
-848
-410
N
DT133
-823
-410
N
DT49
-726
331
Y
DT211
-202
331
Y
DT129
-189
592
Y
DT130
-131
331
Y
DT63
-6
592
Y
DT171
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N
DT150
25
592
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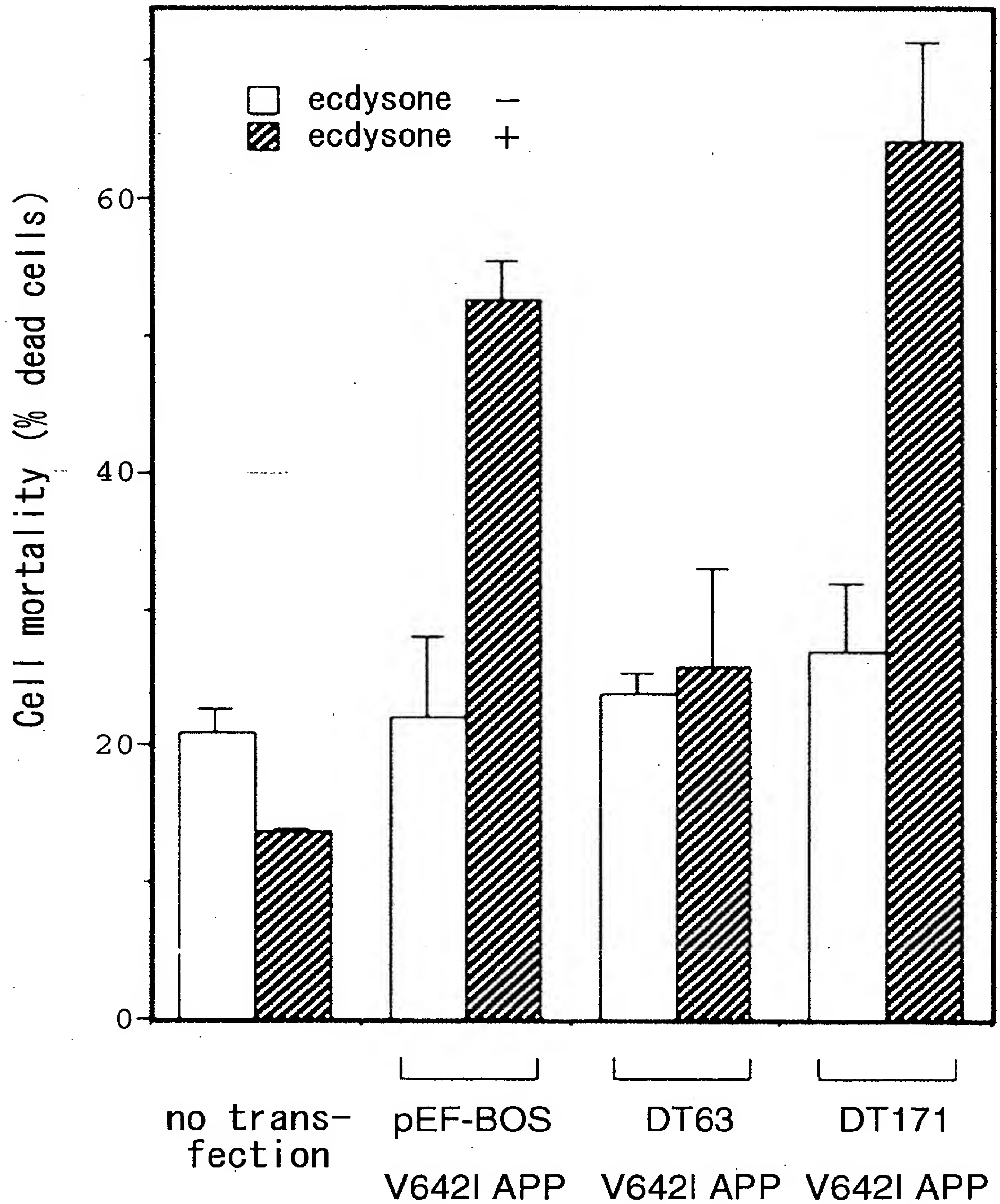
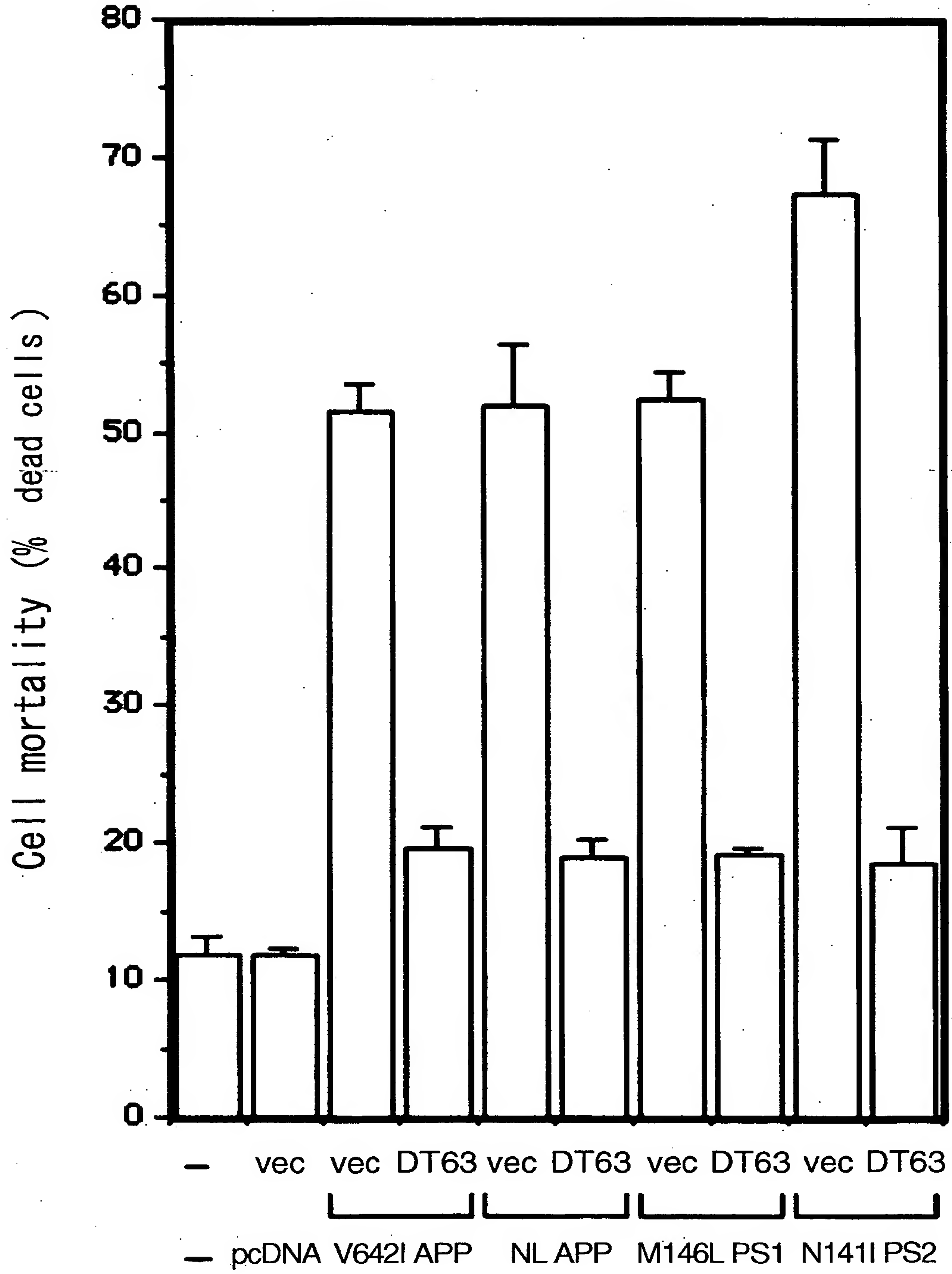
DT207
32
592
N
DT77
184
592
N

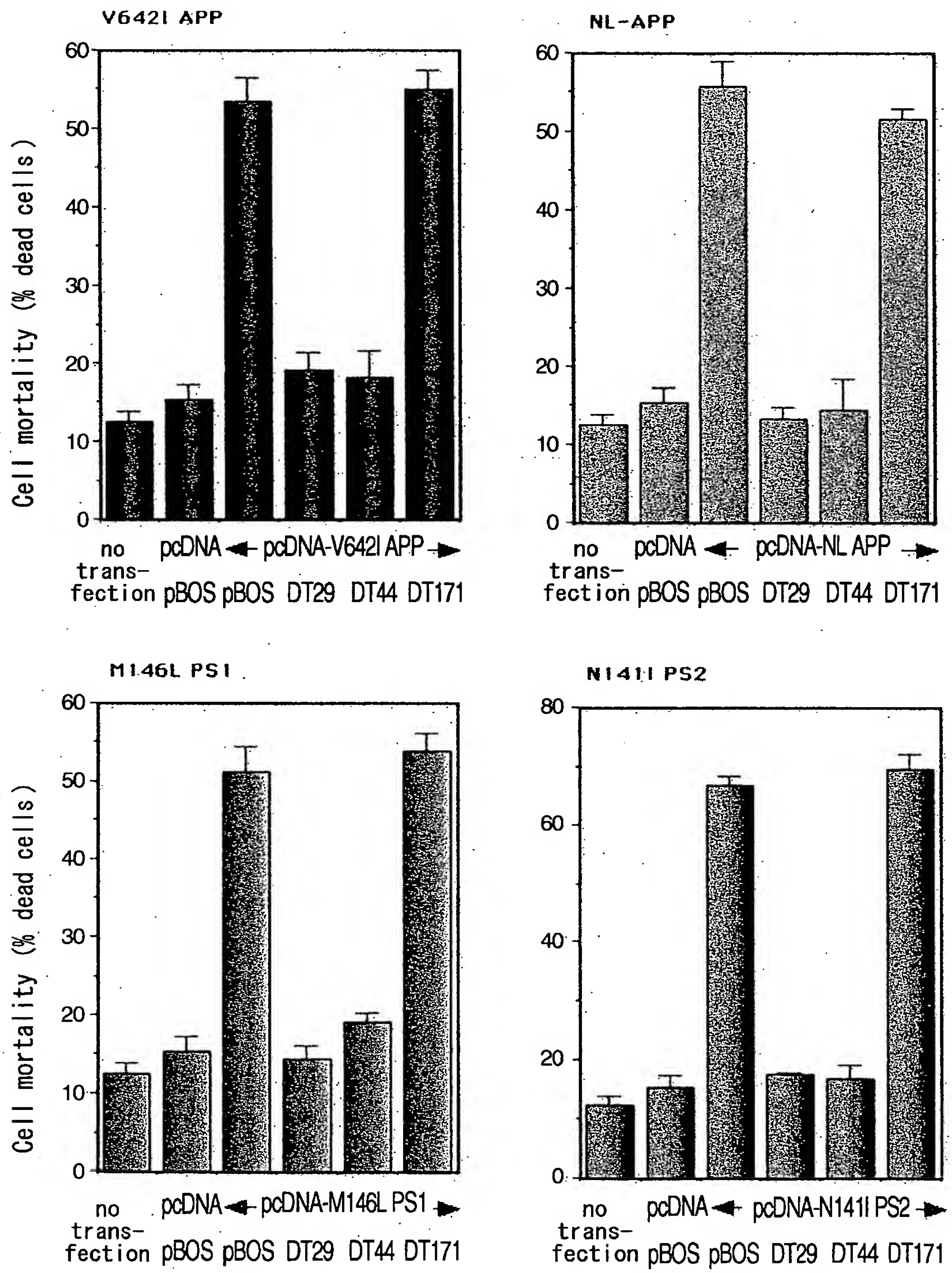
[Figure 2]


ecdysone −
ecdysone +
Cell mortality (% dead cells)
60
40
20
0
no trans-fection
pEF-BOS
V642I APP
DT63
V642I APP
DT171
V642I APP

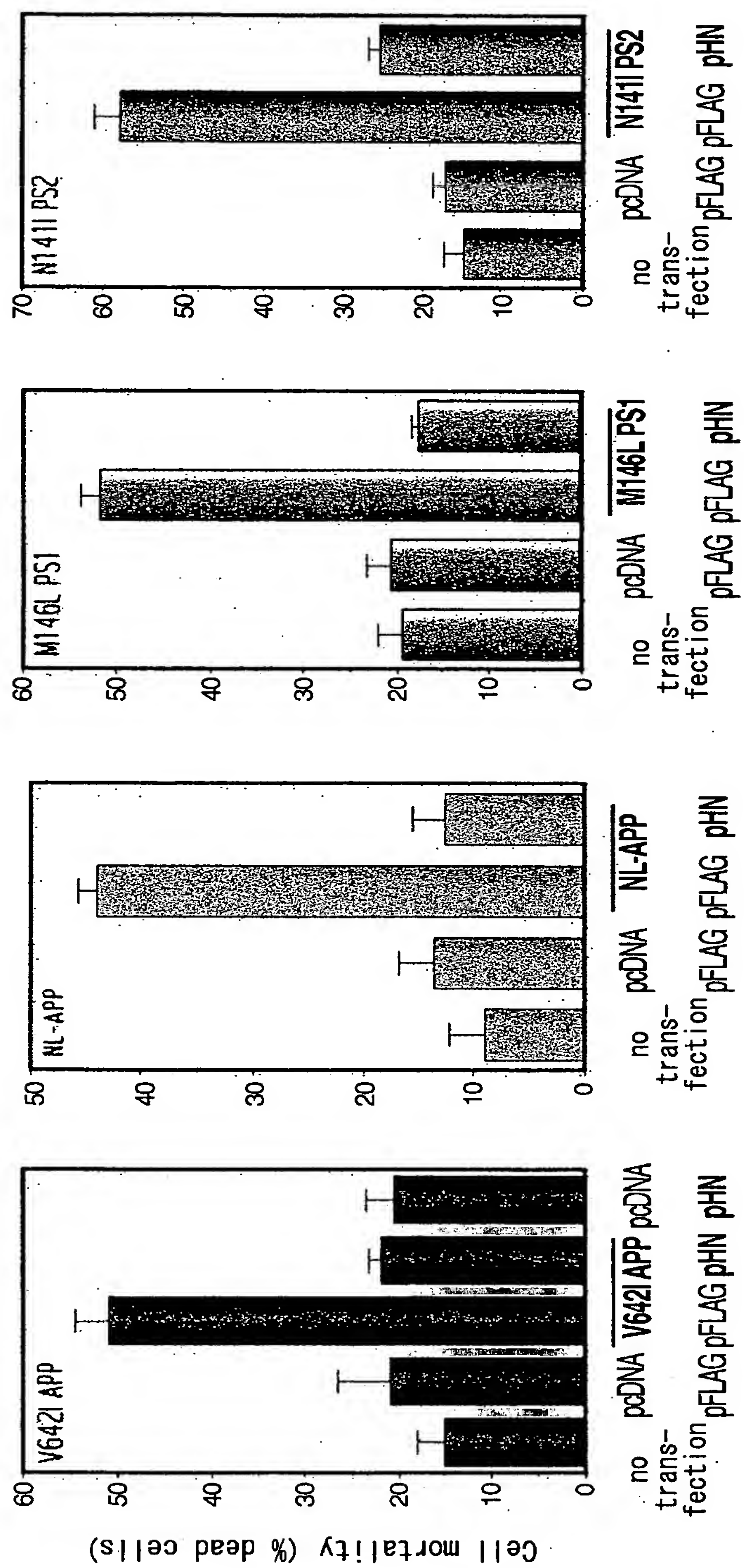
[Figure 3]


Cell mortality (% dead cells)
80
70
60
50
40
30
20
10
0
—
vec
vec
DT63
vec
DT63
vec
DT63
vec
DT63
— pcDNA
V642I APP
NL APP
M146L PS1
N141I PS2

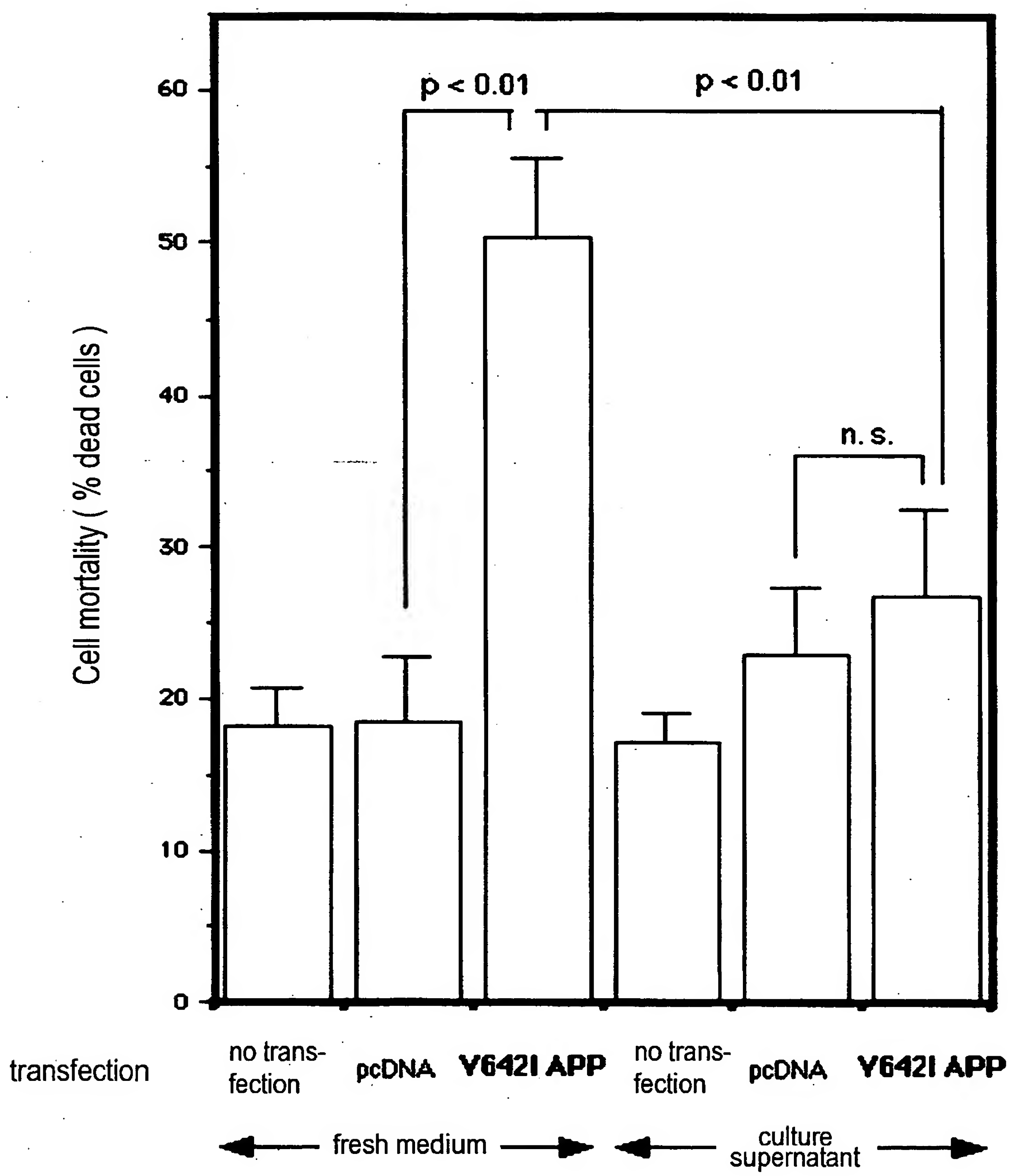
[Figure 4]


V642I APP
NL-APP
M146L PS1
N141I PS2
Cell mortality (% dead cells)
0
10
20
30
40
50
60
80
no trans-fection
pcDNA pBOS
pcDNA-V642I APP
pcDNA-NL APP
pcDNA-M146L PS1
pcDNA-N141I PS2
pBOS
DT29
DT44
DT171

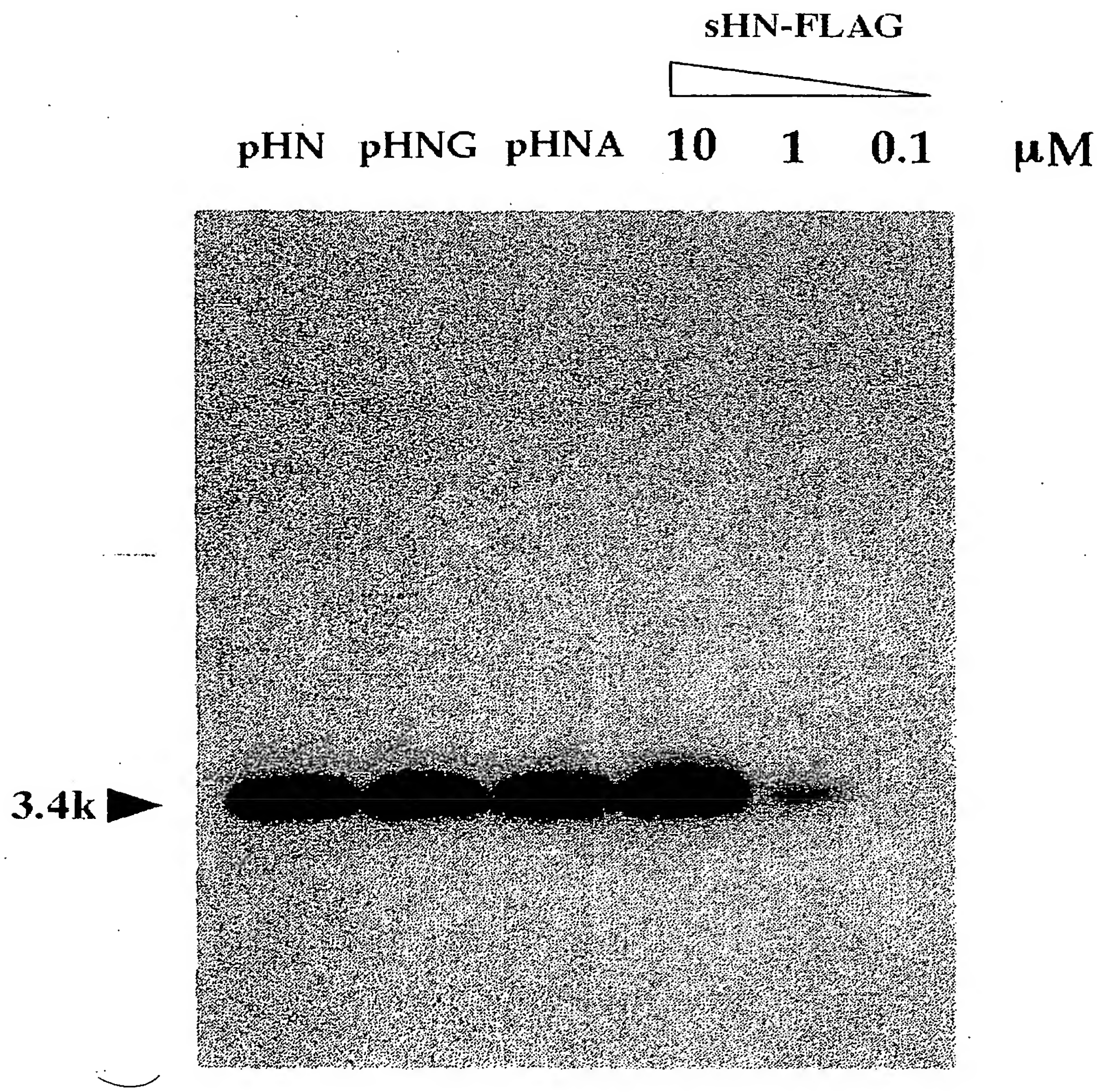
[Figure 5]


V642I APP
NL-APP
M146L PS1
N141I PS2
Cell mortality (% dead cells)
no trans-fection
pcDNA
pFLAG
V642I APP
pFLAG
pHN
pcDNA
pHN
NL-APP
M146L PS1
N141I PS2

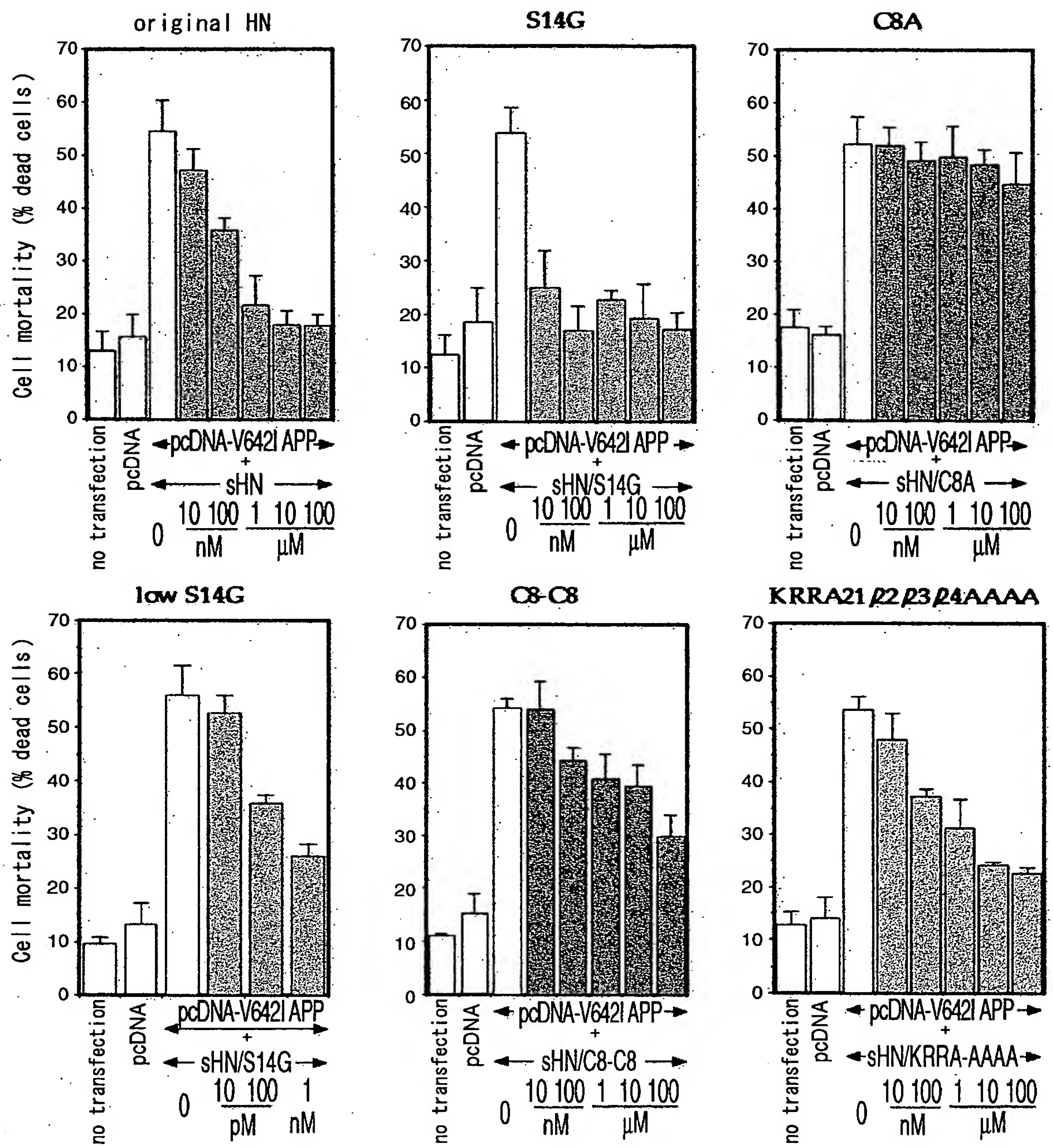
[Figure 6]


Cell mortality (% dead cells)
0
10
20
30
40
50
60
p < 0.01
p < 0.01
n. s.
transfection
no trans-
fection
pcDNA
V642I APP
no trans-
fection
pcDNA
V642I APP
fresh medium
culture
supernatant

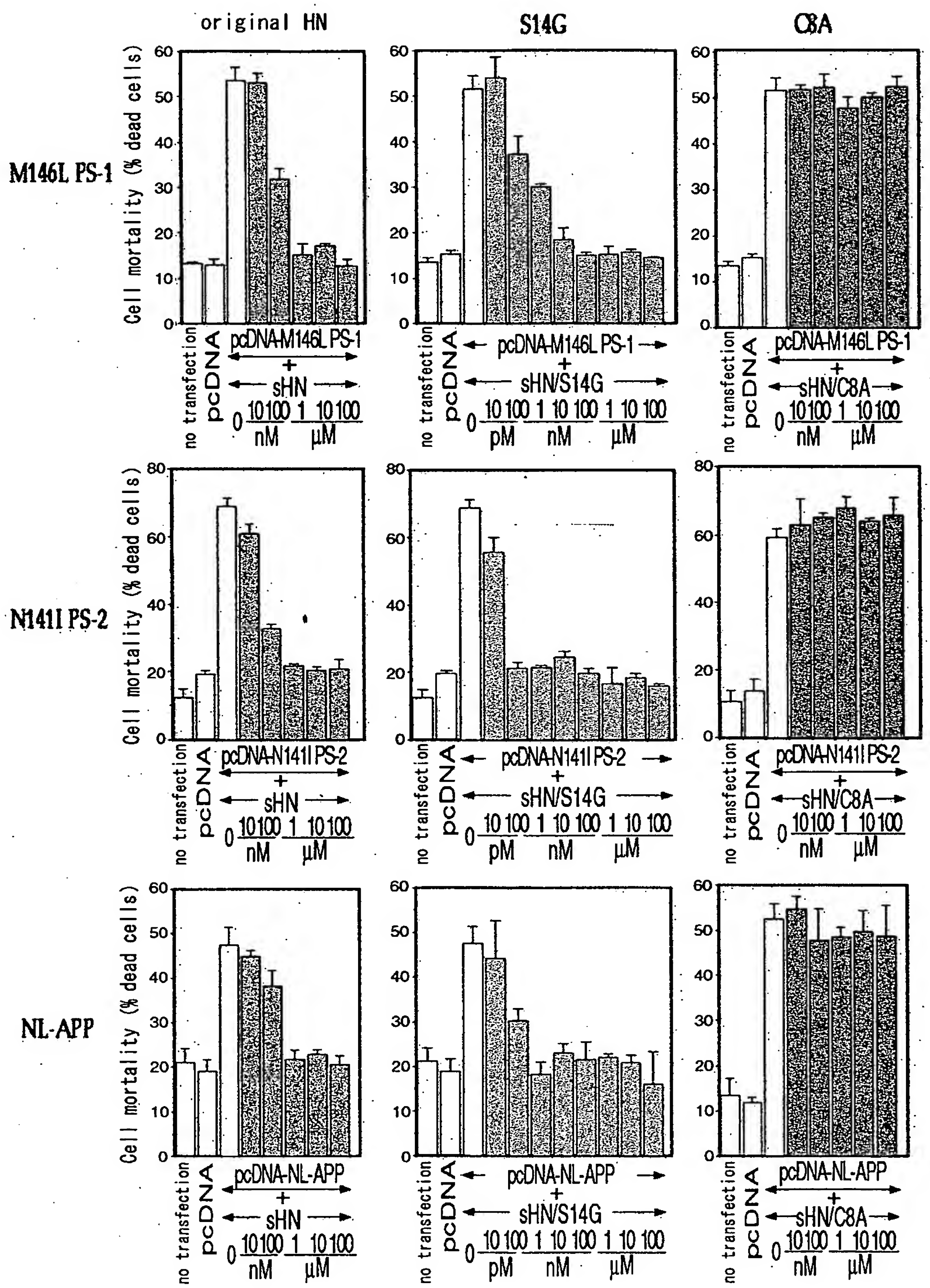
[Figure 7]



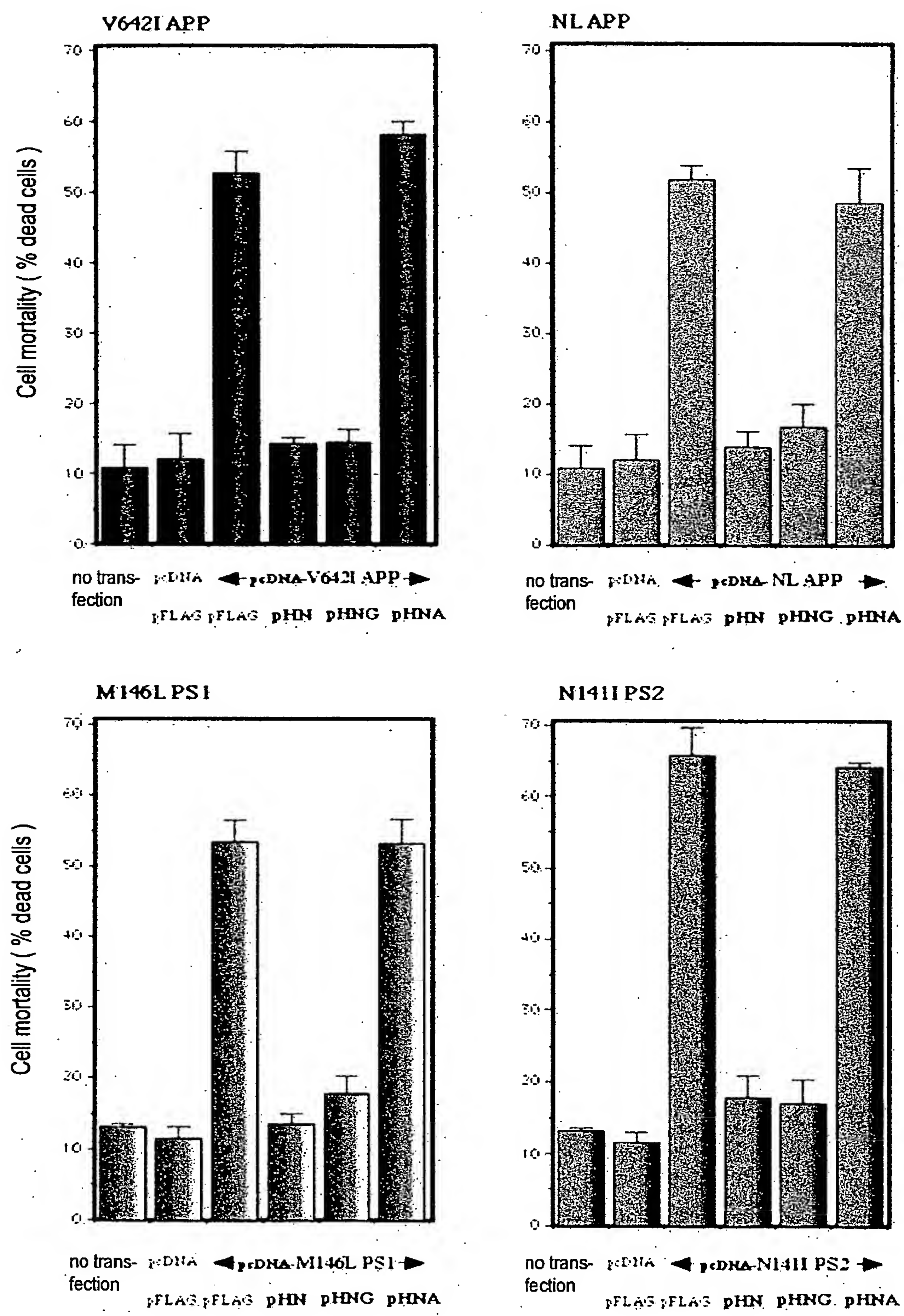
[Figure 8]


original HN
S14G
C8A
low S14G
C8-C8
KRRA21 /22 /23 /24AAAA
Cell mortality (% dead cells)
70
60
50
40
30
20
10
0
no transfection
pcDNA
pcDNA-V642I APP
+
sHN
sHN/S14G
sHN/C8A
sHN/C8-C8
sHN/KRRA-AAAA
0
10 100
1 10 100
nM
μM
10 100
1
pM
nM

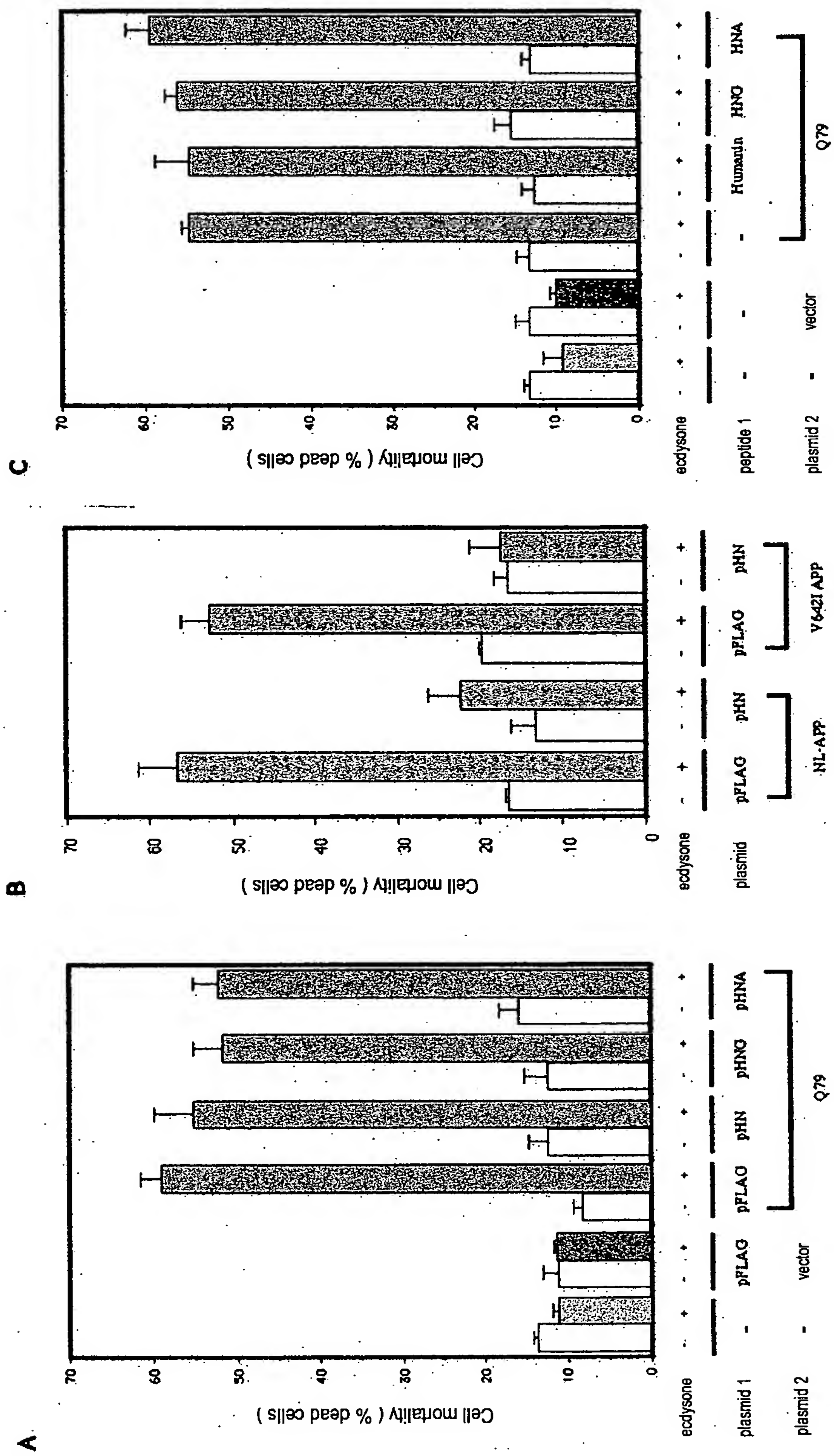
[Figure 9]


original HN
S14G
C8A
M146L PS-1
N141I PS-2
NL-APP
Cell mortality (% dead cells)
no transfection
pcDNA
pcDNA-M146L PS-1
+
sHN
sHN/S14G
sHN/C8A
pcDNA-N141I PS-2
pcDNA-NL-APP
0
10 100 1 10 100
pM
nM
μM

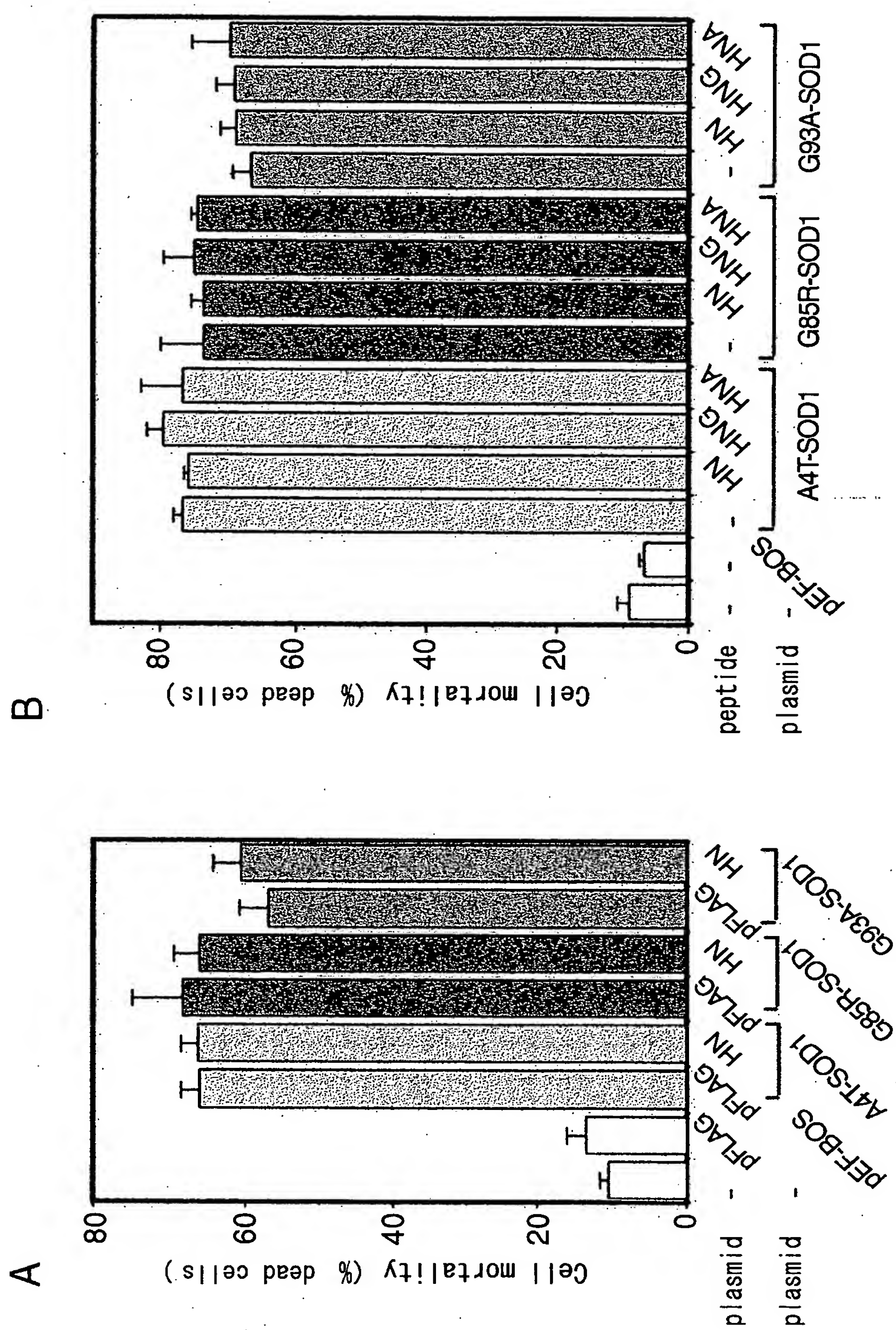
[Figure 10]


V642I APP
NL APP
M146L PS1
N141I PS2
Cell mortality (% dead cells)
Cell mortality (% dead cells)
70
60
50
40
30
20
10
0
no trans-
fection
pcDNA
pcDNA-V642I APP
pFLAG pFLAG pHN pHNG pHNA
no trans-
fection
pcDNA
pcDNA-NL APP
pFLAG pFLAG pHN pHNG pHNA
no trans-
fection
pcDNA
pcDNA-M146L PS1
pFLAG pFLAG pHN pHNG pHNA
no trans-
fection
pcDNA
pcDNA-N141I PS2
pFLAG pFLAG pHN pHNG pHNA

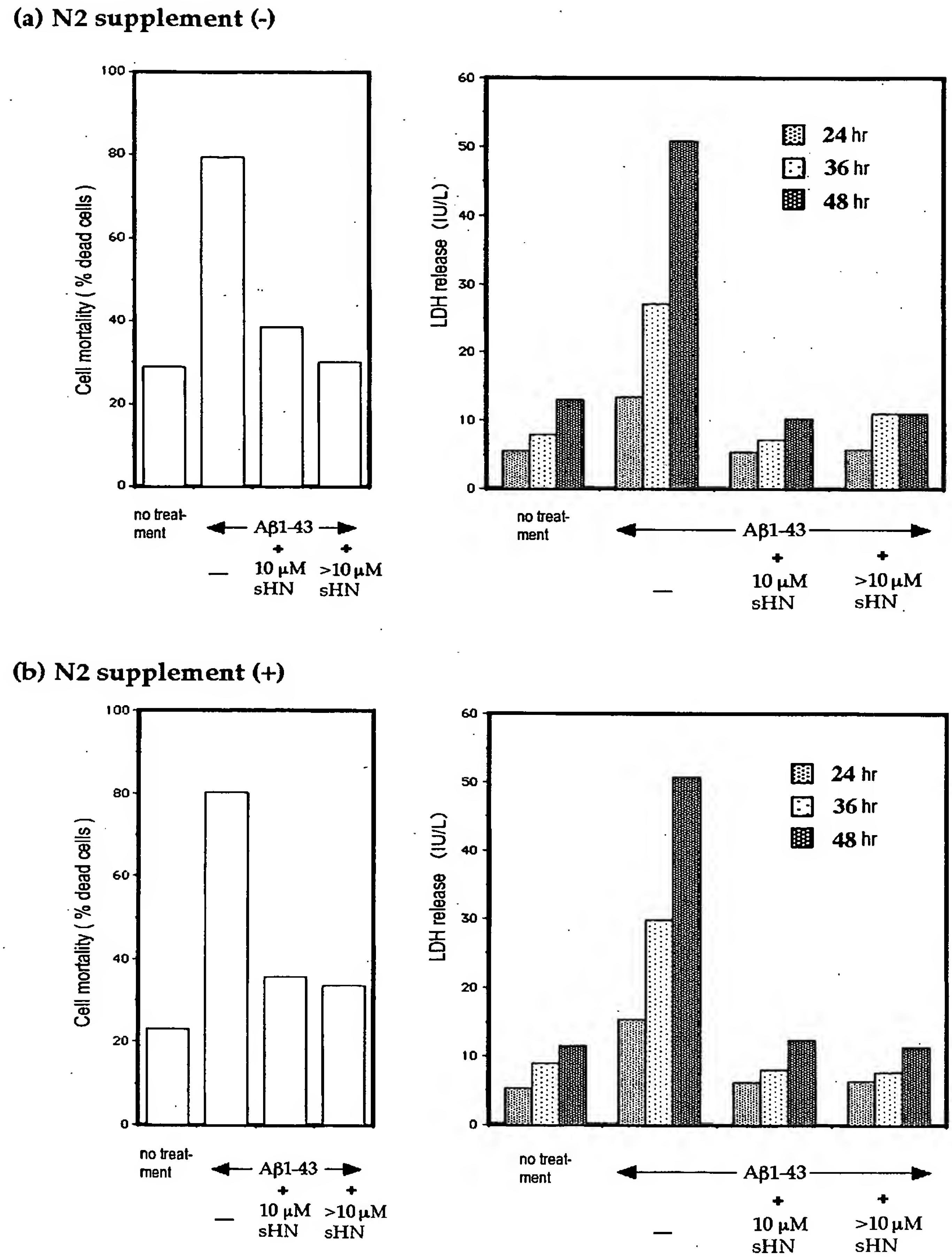
[Figure 11]


A
B
C
Cell mortality (% dead cells)
ecdysone
plasmid 1
plasmid 2
plasmid
peptide 1
pFLAG
pHN
pHNG
pHNA
vector
Q79
NL-APP
V642I APP
Humanin
HNG
HNA

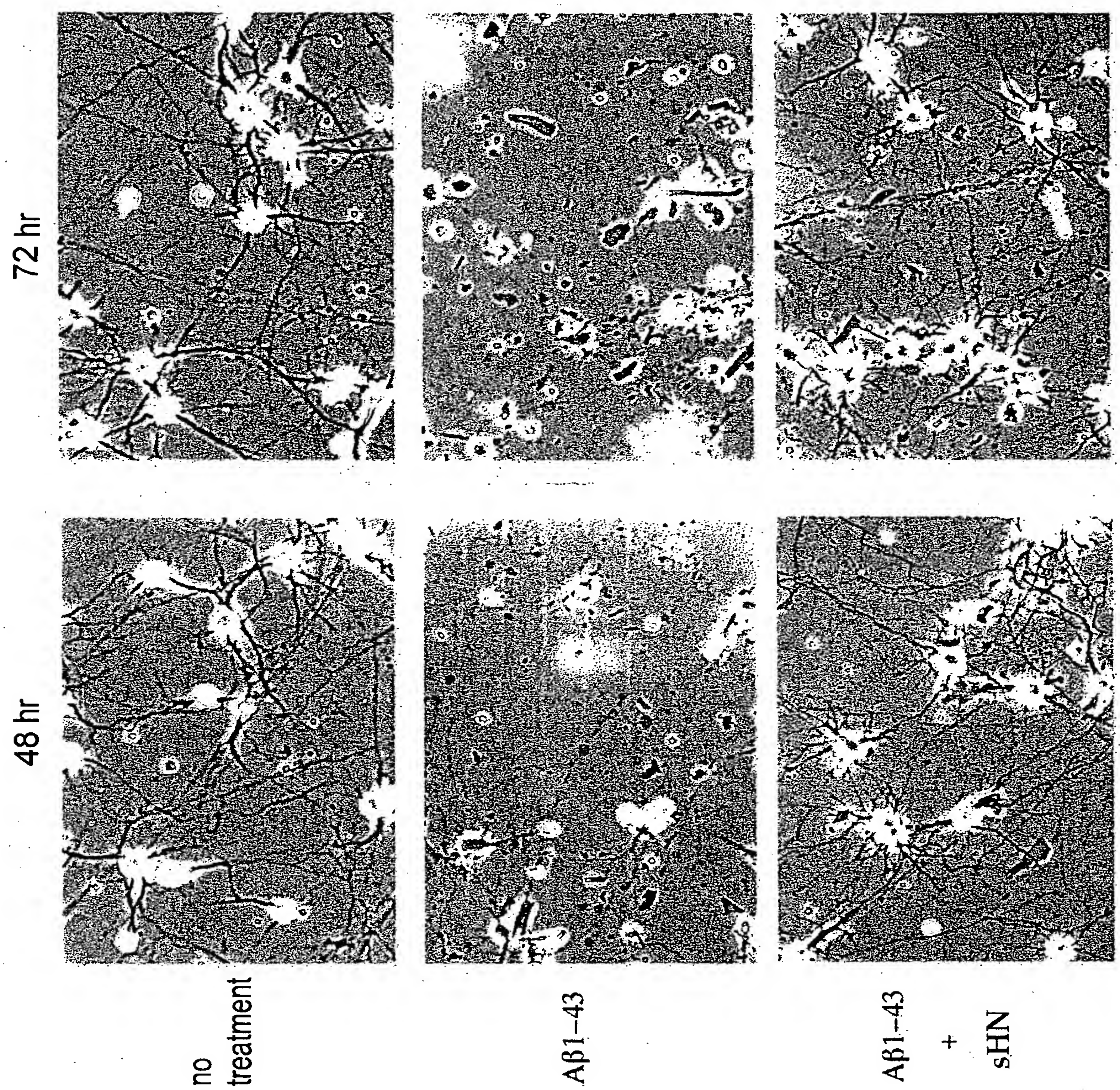
[Figure 12]


A
Cell mortality (% dead cells)
80
60
40
20
0
plasmid
-
pFLAG
pFLAG
HN
pFLAG
HN
pFLAG
HN
plasmid
-
pEF-BOS
A4T-SOD1
G85R-SOD1
G93A-SOD1
B
Cell mortality (% dead cells)
80
60
40
20
0
peptide
-
-
-
HN
HNG
HNA
-
HN
HNG
HNA
-
HN
HNG
HNA
plasmid
-
pEF-BOS
A4T-SOD1
G85R-SOD1
G93A-SOD1

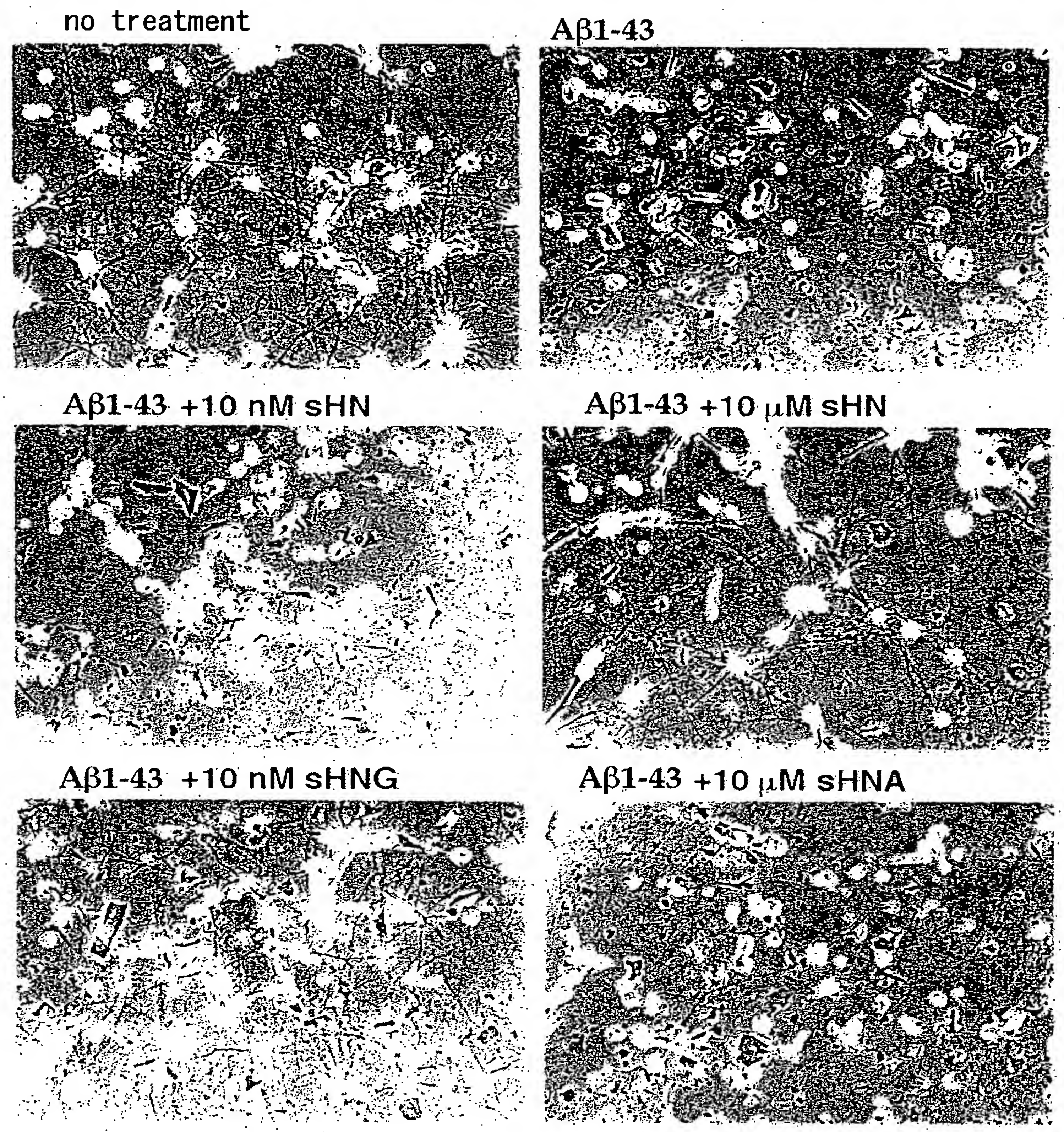
[Figure 13]


(a) N2 supplement (-)
Cell mortality (% dead cells)
0
20
40
60
80
100
no treat-ment
Aβ1-43
—
+
10 μM sHN
+
>10 μM sHN
LDH release (IU/L)
0
10
20
30
40
50
60
24 hr
36 hr
48 hr
no treat-ment
Aβ1-43
—
+
10 μM sHN
+
>10 μM sHN
(b) N2 supplement (+)
Cell mortality (% dead cells)
0
20
40
60
80
100
no treat-ment
Aβ1-43
—
+
10 μM sHN
+
>10 μM sHN
LDH release (IU/L)
0
10
20
30
40
50
60
24 hr
36 hr
48 hr
no treat-ment
Aβ1-43
—
+
10 μM sHN
+
>10 μM sHN

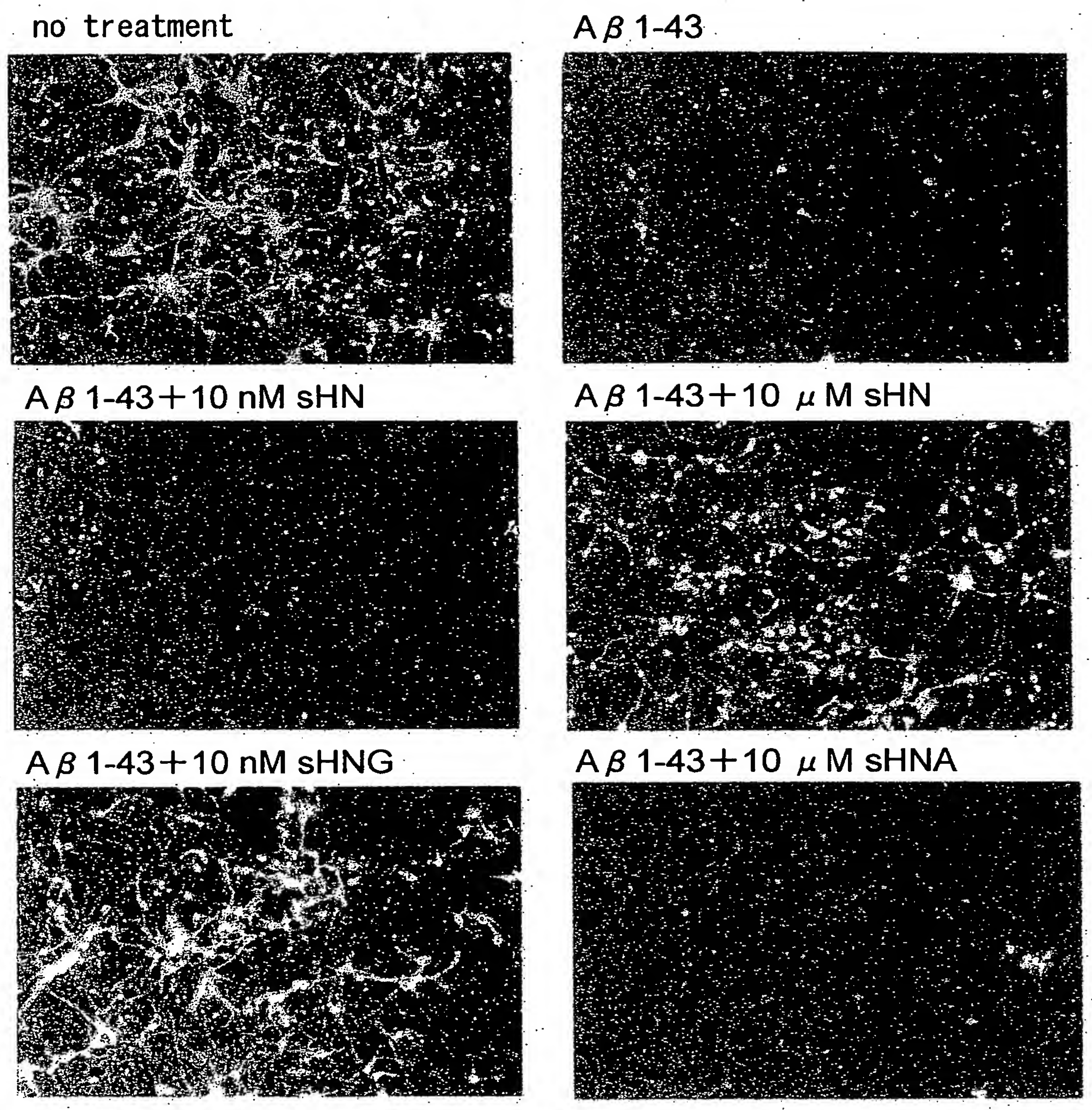
[Figure 14]



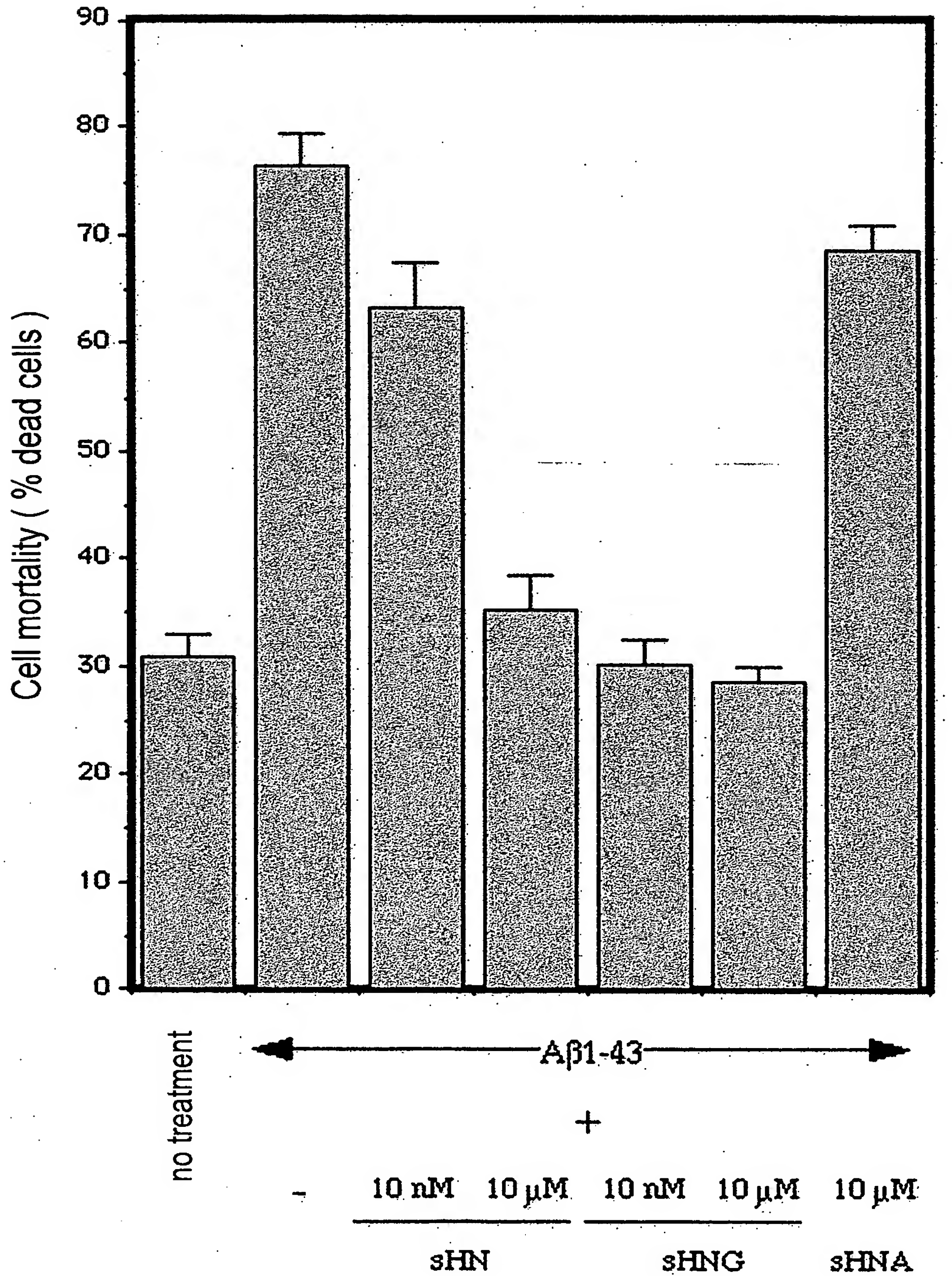
[Figure 15]


no treatment
Aβ1-43
Aβ1-43 +10 nM sHN
Aβ1-43 +10 μM sHN
Aβ1-43 +10 nM sHNG
Aβ1-43 +10 μM sHNA

[Figure 16]


no treatment
Aβ 1-43
Aβ 1-43+10 nM sHN
Aβ 1-43+10 μM sHN
Aβ 1-43+10 nM sHNG
Aβ 1-43+10 μM sHNA

[Figure 17]


90
80
70
60
50
40
30
20
10
0
Cell mortality (% dead cells)
no treatment
Aβ1-43
+
-
10 nM
10 μM
10 nM
10 μM
10 μM
sHN
sHNG
sHNA

[Document Name] Abstract

[Abstract]

[Problems to Be Solved] The present invention provides polypeptides that suppresse neuronal death associated with Alzheimer's disease.

[Means to Solve the Problems] Using a neuronal cell system, wherein the expression of familial Alzheimer's disease mutant APP can be induced by ecdysone treatment, a gene that protects the neurons from cell death was successfully isolated. The gene encodes a secretory polypeptide consisting of 24 amino acids, and this polypeptide suppresses neuronal death caused by the expression of APP mutants and presenilin mutants. The polypeptide also suppressed cell death of primary neuronal culture caused by Aβ. Furthermore, by substituting the amino acids of the polypeptide, the neuronal death suppression activity of the polypwptide was successfully and significantly enhanced. These polypeptides and derivatives thereof are useful as pharamaceuticals to prevent neuronal death associated with Alzheimer's disease, and as seed compounds for developing novel pharmaceuticals for Alzheimer's disease.

[Selected Drawings] None